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DÉVELOPPEMENT DE MICRO-SYSTÈME IMPRÉGNÉ DE BIOCHARBON-ENZYME (BEMS) POUR LA DÉGRADATION DU CONTAMINANT ÉMERGENT – DICLOFÉNAC

Par

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DÉDICACE

To my mother for her love, sacrifices, endless motivation and support

In memory of my late father and grandparents

To all my teachers

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RÉSUMÉ

Les contaminants émergents attirent de plus en plus l'attention en raison de leur omniprésence dans les milieux naturels, leur impact sur l'environnement ainsi que de leurs effets toxiques sur divers organismes. Le Diclofénac (DCF) est un médicament anti-inflammatoire largement utilisé dans le monde entier. Plusieurs recherches menées au cours des dernières décennies ont confirmé l'ubiquité mondiale de DCF dans l'environnement. Pour le DCF, 30 à 70% sont éliminées par le système de traitement classique dans les stations d'épuration des eaux usées (qui est le principal récepteur primaire du contaminant). Ainsi, le DCF résiduel a été fréquemment détecté dans les eaux douces et connu et sa toxicité a été observée pour plusieurs organismes aquatiques. À titre d'exemple, à une concentration de 1 µg L^{-1}, le DCF induits des altérations physiologiques et cause des dommages aux reins et au foie. Le développement de méthodes de traitement efficace des composés pharmaceutiques est donc nécessaire.

L'identification et la quantification des DCF au Québec au niveau de la station de traitement des eaux usées de la Communauté urbaine (ville de Québec, Canada) est une étape clef pour étudier le devenir du DCF dans l'environnement. A cet effet, un procédé rapide et indépendant de la matrice a été développé en utilisant LDTD-APCI-MS / MS (ionisation chimique à pression atmosphérique désorption thermique diode laser (LDTD-APCI) couplée à une spectrométrie de masse tandem (MS / MS). Le procédé réduit le temps d'analyse par échantillon à 12 secondes comparativement à la méthode conventionnelle LC-ESI-MS / MS qui prend jusqu'à 12 minutes. Les eaux usées non traitées contenaient 64,89 ± 6.7µg L^{-1} de DCF et l'effluent contenait 15,95 ± 3.7µ g L^{-1}. La quantification du DCF dans les boues des eaux usées a également été réalisée et pour lesquelles, deux méthodes d'extraction ont été étudiées. L'extraction par solvant accélérée (ASE) a été la plus efficace comparée à l'extraction assistée par ultrasons (USE) avec 95,6 ± 7% de récupération. Les boues primaires (1,10 ± 0,15 µg g^{-1}) et boues secondaires (0,90 ± 0,15 µg g^{-1}) contenaient des

concentrations importantes de DCF et qui est en outre dirigé vers la sorption du DCF dans les eaux usées. Dans l'ensemble, un enlèvement de près de 76% du DCF a été observé dans la station d'épuration des eaux usées. Cependant, une grande partie de cet enlèvement est due à l'adsorption du contaminant sur les boues des eaux usées. Après avoir déterminé la répartition du DCF tout au long des différentes opérations unitaires de la station d'épuration, il était important de mettre au point des méthodes de traitement.

Le traitement par adsorption a été proposé comme étant un procédé d'élimination efficace de divers contaminants émergents. Dans cette étude, les microparticules de biochars ont été produites à partir du biochar obtenu par broyage et tamisage. Les propriétés du biochar, ainsi que ses capacités d'adsorption sont fortement dépendantes du choix de la matière première agricole utilisée et des conditions de sa production. Les microbiochars ont été caractérisés pour leurs capacités d'adsorption en utilisant le bleu de méthylène comme contaminant organique modèle. Pour le DCF, le microbiochar produit à partir de fumier de porc (BC-PM) a montré d'excellente efficacité d'élimination (99,6%) comparativement à celui obtenu à partir de bois de pin (BC-PW). La diffusion intraparticulaire est considérée l'élément limitant dans le processus d'adsorption. La température, la présence d'ions co-existants, la concentration initiale d'adsorbat et les particules ont joué tous un rôle important dans l'adsorption.

Afin d'améliorer leurs capacités d'adsorption pour le DCF, les microbiochars ont été fonctionnalisées en utilisant divers acides organiques. La fonctionnalisation a augmenté les groupes fonctionnels acides de 23,6%, 10,2% et 26,2%, respectivement pour le bois de pin, le fumier de porc et les microbiochars de coquille d'amande. Les biochars fonctionnalisés à l'acide citrique avaient la capacité la plus élevée pour éliminer le DCF. Pour le biochar à base de bois de pin, l'équilibre est passé de 65 à 80% lors du traitement des eaux contaminées avec le DCF Pour le biochar à base de coquilles d'amande (BC-AS) et dans des conditions similaires, l'efficacité d'élimination de diclofénac est passée de 58 à 84%.

Les enzymes lignolytiques, particulièrement la laccase en, a été démontrée efficace pour la dégradation de plusieurs contaminants émergents. Des essais ont été menés pour une production rentable de laccase via l'utilisation des résidus agro-industriels comme substrats. Les résidus d'industrie de jus de fruit (49.16 ± 2,5 U gds $^{-1}$) et de l'industrie de pâte et papier (52,4 ± 2,2 U gds^{-1}) se sont avérés être des substrats efficaces comparés aux fibres d'alfa séchées (14,26 ± 0,8 U gds^{-1}). Une dégradation presque totale (99%) a été obtenue après une période de 5 heures lorsque le pH et la température sont fixés respectivement à 4,5 et à 50ºC. L'immobilisation de la laccase sur des supports solides permet d'améliorer sa stabilité et de renforcer le potentiel d'application dans des conditions environnementales réelles. Les procédés d'immobilisation par adsorption et covalente ont été étudiés en utilisant de la laccase brute sur divers microbiochars. Avec tous les types de biochars, et comme l'activité initiale de la laccase est élevée dans la solution brute, l'efficacité de l'immobilisation était élevée aussi. Le modèle qui décrit le mieux à liaison enzymes-micro-biochars est la forme d'une monocouche homogène La laccase immobilisée par des liaisons covalentes a montré une stabilité supérieure à différentes conditions (pH, température et durée de stockage). Avec l'immobilisation covalente de la laccase en utilisant du glutaraldéhyde (5% v / v), le microbiochar à base de lisier de porc a montré l'efficacité d'adsorption la plus élevée (34,1 ± 1. 1 U g^{-1}), suivie par microbiochar de coquille d'amande (25,3 ± 2,8 U g^{-1}) et celui de bois de pin (16,18 ± 0,3 U g^{-1}). En outre, le prétraitement avec de l'acide citrique améliore les interactions de la laccase avec tous les microbiochars et a montré une amélioration particulière de l'ordre de 20% pour le microbiochar à base de coquille d'amandes. À une concentration de 500 µgL^{-1}, l'élimination totale du DCF a été observée avec le complexe micro biochar de lisier de porc-laccase au bout de 3 heures. Plus de 40% de l'activité de la laccase a été maintenue avec tous les systèmes micro-biochars-laccase-après 5 cycles de traitement de DCF d'une durée 6 heures.

Les nouveaux microsystèmes imprégnés biochar-enzyme laccase (BEMS) ont été exploités pour la fabrication d'un bioréacteur à colonne sur lit fixe. Le biochar-la laccase a démontré

une efficacité de traitement plus élevé que le biochar brut. De plus, le biochar-laccase a permis une amélioration significative pour le temps de résidence grâce à la biodégradation en parallèle du DCF dans la colonne. Le microsystème biochar imprégné d'enzyme (BEMS) est une approche efficace, durable, verte et rentable pour le traitement des DCF et d'autres contaminants émergents dans divers environnements.

ABSTRACT

Emerging contaminants are gaining wide attention due to their omnipresence in environmental compartments and their potential environmental as well as toxic effects on various organisms. Diclofenac (DCF) is a prevalent anti-inflammatory drug used throughout the world. Intensive researches carried out in the past few decades have confirmed the global ubiquity of DCF in various environmental compartments. For DCF, about 30–70% removal has been obtained through the conventional treatment system in wastewater treatment plant is the major primary sink. Thus, the untreated DCF has frequent occurrence in freshwater environments and known for its potential toxicity towards several aquatic organisms. At 1 µg L^{-1}, DCF induces physiological alterations and kidney and liver damages in some aquatic organisms and which call up for effective treatment methods for the removal of the pharmaceuticals.

The identification and quantification of DCF in Quebec Urban Community wastewater treatment (Quebec city, Canada) was performed to study the fate of DCF in the environment. For this purpose, a rapid and less matrix-prone method was developed using LDTD-APCI-MS/MS (laser diode thermal desorption-atmospheric pressure chemical ionization (LDTD-APCI) coupled to tandem mass spectrometry (MS/MS). The method reduced the analysis time per sample to 12 seconds when compared with the conventional LC-ESI–MS/MS method (12 minutes). The wastewater influent contained 64.89±6.7µg L^{-1} of DCF and the effluent contained 15.95±3.7µg L^{-1}. Quantification of DCF in wastewater sludge was also carried out and for the which, two extraction methods were studied. Accelerated solvent extraction (ASE) was found to be effective over ultrasonic-assisted extraction (USE) for the extraction of DCF from wastewater sludge with 95.6 ± 7% recovery. Primary sludge (1.10 ± 0.15 µg g^{-1}) and secondary sludge (0.90 ± 0.15 µg g^{-1}) contained significant amounts of DCF and which further pointed towards the sorption of DCF in wastewater sludge. Overall, nearly 76 % removal of DCF was observed in the wastewater treatment plant; however, a

major portion of this was accounted through sorption onto the wastewater sludge. After having determined the partitioning of DCF in different wastewater treatment plant unit operations, it was important to devise treatment methods.

Adsorptive removal has been recognized as an effective removal method for various emerging contaminants. In this study, the micro-biochars were produced from the biochar obtained from pyrolysis reactor outlet through size reduction. Feedstock selection and method of production had significant effects on the biochar properties as well as adsorption capacities. Micro-biochars were characterized for the adsorption experiments using the model organic contaminant methylene blue. For emerging contaminant DCF, Pig manure micro-biochar (BC-PM) showed excellent removal efficiency (99.6%) over pine wood micro-biochar (BC-PW) at 500 µg L^{-1} of DCF (environmentally significant concentration). Intraparticle diffusion was found to be the major process facilitated the adsorption. Thermodynamics, the presence of co-existing ions, initial adsorbate concentration and particles size played a substantial role in adsorption. Further, the micro-biochars were functionalized using various organic acids for enhanced removal of DCF. The functionalization increased total acidic functional groups by 23.6 %, 10.2 %, and 26.2 %, respectively for pine wood, pig manure, and almond shell micro-biochars. Citric acid functionalized biochars showed the higher removal of the environmentally relevant concentration (500 µg L^{-1}) of emerging contaminant, diclofenac. For pinewood biochar, at equilibrium diclofenac removal increased from 65 to 80 %. For almond shell biochar (BC-AS), under similar conditions, the diclofenac removal efficiency increased from 58 to 84 %.

Ligninolytic enzymes, laccase, in particular, were found to be effective for the degradation of several emerging contaminants. In this study, an attempt has been made for the cost-effective production of laccase through the application of agro-industrial residues as substrates (using *Tremetes versicolor* (ATCC 20869)*).* Apple juice industry residue, apple pomace (49.16±2.5 U gds^{-1}) and pulp and paper solid waste (52.4±2.2 U gds^{-1}) were found to be the efficient substrates for laccase production over dried alfa fibers (14.26±0.8 U gds^{-1}).

At environmentally relevant concentrations (500 µg L^{-1} of DCF), pH of 4.5 and temperature of 50 °C was found to be optimal for the effective degradation of DCF with laccase. Under optimized conditions, up to 99 % degradation was observed for DCF within 5 hours. Immobilization of laccase on solid supports will enhance its stability and further application potential in real environmental conditions. Adsorptive and covalent immobilization methods were studied for the immobilization of crude laccase on various micro-biochars. With all biochars, as the initial activity of laccase increased in the crude solution, the adsorptive immobilization efficiency also increased. Homogeneous monolayer adsorption was found to be the major mechanism of enzyme binding on micro-biochars. Covalently immobilized laccase (using glutaraldehyde as cross linker) showed superior pH, thermal, storage and operational stability. With covalent immobilization of laccase using glutaraldehyde (5% w/v), pig manure micro-biochar showed highest laccase binding (34.1±1.1 U g^{-1}) followed by almond shell micro-biochar (25.3±2.8 U g^{-1}) and pine wood micro-biochar (16.18±0.3 U g^{-1}). Further, citric acid pretreatment improved the laccase binding capacity of all the micro-biochars and showed up to 20 % improvement in laccase binding with almond shell micro-biochar. At environmentally relevant concentration (500 µg L-1), the complete removal of DCF was observed with pig manure micro biochar bound laccase within 3 hours under batch mode operating conditions. More than 40 % of the laccase activity was retained with all the laccase-bound micro-biochars after 5 cycles of diclofenac treatment in which each cycle lasted for 6 hours.

Laccase-bound micro-biochars were developed into novel biochar-enzyme impregnated microsystem (BEMS) by developing a fixed-bed column bioreactor. Laccase bound biochar exhibited higher removal efficiency over the raw biochar and significant improvement in breakthrough time was observed which was attributed to the biodegradation of DCF in the column. The biochar-enzyme impregnated microsystem (BEMS) will be promising as an efficient, sustainable, green and cost-effective approach for the treatment of DCF and other emerging contaminants in diverse environments.

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TABLE DES MATIÈRES

LISTE DES TABLEAUX

CHAPTER 1

CHAPTER 2

PART 1

PART 2

CHAPTER 3

PART 1

PART 2

LISTE DES FIGURES

CHAPTER 1

CHAPTER 2

PART 1

PART 2

CHAPTER 3

PART 1

PART 2

PART 3

PART 4

CHAPTER 4

PART 1

PART 2

PART 3

CHAPTER 5

LISTE DES ABRÉVIATIONS

ANOVA	Analysis of variance
AOP	Advanced oxidation processes
APCI	Atmospheric pressure chemical ionization
ASE	Accelerated solvent extraction
BC-AS	Biochar- almond shell
BC-PM	Biochar- pig manure
BC-PW	Biochar- pine wood
BEMS	Biochar-enzyme impregnated microsystem
BET	Brunauer, Emmett and Teller
DCF	Diclofenac
EC	Emerging contaminant
EC_{50}	Effective concentration- half maximal
EDTA	Ethylenediaminetetraacetic acid
EMA	European medicines agency
EML	Emergency medical list
EQS	Environmental quality standards
ESI	Electrospray ionization
FTIR	Fourier transform infrared spectroscopy
HRT	Hydraulic retention time
IMS	Intercontinental Marketing Services
IUCN	International union for conservation of nature

IUPAC	International union of pure and applied chemistry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDTD-MS/MS	Laser diode thermal desorption-tandem mass spectrometry
LME	Lignin-modifying enzyme
LPO	Lipid peroxidation
MB	Methylene blue
MBR	Membrane bioreactor
MLSS	Mixed liquor suspended solids
MWCNT	Multiwalled carbon nanotubes
NSAID	Non-steroidal anti-inflammatory drug
PhAC	pharmaceutically active compound
SEM	Scanning electron microscope
SRT	Sedimentation retention Time
USE	Ultrasonic extraction
WW	Wastewater
WWS	Wastewater sludge
WWTP	Wastewater treatment plant
XRD	X-ray diffraction
YES	Yeast estrogen screen

CHAPTER 1

SYNTHÈSE

PARTIE 1: INTRODUCTION

Les contaminants d'intérêt émergent (CEC) ont attiré l'attention au cours des dernières décennies en raison de leurs impacts négatifs sur les humains et l`écosystème. Les produits pharmaceutiques et de soins personnels (PPCPs) représentent un large éventail de CECs et sont de plus en plus détectés dans le milieu aquatique. Les composés pharmaceutiquement actifs (PhACs) forment l'une des classes les plus remarquables des produits pharmaceutiques qui, par une voie ou par une autre, entrent dans l'environnement en tant que composé parent ou en tant que métabolites pharmacologiquement actifs. Parmi les PhACs, les anti-inflammatoires non stéroïdiens (NSAIDs) sont largement utilisés dans le monde et détectés dans différents compartiments environnementaux à des concentrations allant de ng L^{-1} à mg L^{-1} (Collins and Dobson, 1997; Halling-Sørensen et al., 1998).

Le diclofénac (DCF), souvent reconnu comme l'analgésique le plus populaire au monde, est également le médicament anti-inflammatoire non stéroïdien le plus utilisé (NSAID), avec une part de marché proche de celle des trois médicaments les plus populaires (ibuprofène, méfénamique acide, naproxène) (McGettigan and Henry, 2013). Des estimations récentes font état d'une consommation mondiale de DCF de 1443 ± 58 tonnes/an (Acuña et al., 2015). Habituellement, les médicaments sont développés dans l'intention d'avoir un effet biologique bénéfique sur l'organisme auquel ils sont administrés, bien que beaucoup de ces composés passent souvent dans l'environnement où ils peuvent exercer un effet biologique indésirable (Halling-Sørensen et al., 1998).

Souvent, le DCF n'est pas complètement éliminé des usines de traitement des eaux usées (WWTP) en raison de sa faible dégradation et de ses taux de consommation plus élevés (Fatta-Kassinos et al., 2011; Zorita et al., 2009). Par conséquent, le DCF est fréquemment détecté dans les eaux de surface, les sédiments et les boues (Kunkel and Radke, 2012; Langford et al., 2011). Relativement récemment, le DCF a attiré davantage l'attention en raison de sa présence fréquente dans les sources d'eau potable et de ses effets nocifs sur de nombreux organismes à des concentrations

significatives (Cleuvers, 2004; Oaks et al., 2004). Les systèmes conventionnels de traitement des eaux usées ont obtenu une efficacité d'élimination modérée à élever pour le DCF. L'efficacité d'élimination du système conventionnel de traitement des eaux usées pour le diclofénac variait entre 30 et 70% et une grande partie de cette élimination est comptabilisée par l'adsorption sur les boues (Vieno and Sillanpää, 2014; Zhang et al., 2008). Cependant, des concentrations de DCF aussi basses que 1 µg L^{-1} peuvent entraîner des dommages environnementaux, tel que l'effet toxique envers certaines espèces de poissons (Schwaiger et al., 2004; Triebskorn et al., 2004). Par conséquent, il est nécessaire d'éliminer complètement et efficacement le DCF et ses résidus de l'environnement.

Au cours des dernières décennies, l'adsorption est apparue comme une méthode de traitement efficace pour l'élimination de divers contaminants, y compris les composés organiques traces (contaminants émergents). Récemment, le biochar est devenu un adsorbant efficace et rentable pour divers contaminants émergents. La structure poreuse et les caractéristiques chimiques de surface, telles que la présence de divers groupes fonctionnels, font du biochar un excellent adsorbant pour les PhAC’s et DCF en particulier. Les enzymes ligninolytiques, la laccase en particulier, se sont également avérées efficaces pour le traitement de plusieurs contaminants émergents (Gassara et al., 2013b). Plusieurs procédés d'oxydation avancés (AOPs) sont actuellement appliqués pour le traitement des contaminants émergents. Cependant, ces processus peuvent produire des produits de transformation qui peuvent être encore plus toxiques que le composé d'origine. Malgré tout, il est peu probable qu'un traitement enzymatique conduise à la formation d'un produit de transformation toxique. Dans les deux cas, une élucidation plus poussée de la nature des produits de dégradation est nécessaire pour évaluer l'efficacité globale du traitement.

Dans ce projet de recherche, un procédé hybride a été développé en immobilisant des enzymes sur des microbiochars pour l'élimination du DCF. Cette thèse a été divisée en quatre sections principales. La première section étudie l'occurrence, le devenir et la toxicité du DCF. En outre, la

sortie du DCF a été étudiée dans une station d'épuration conventionnelle et une nouvelle méthode a été développée pour la quantification du DCF dans les eaux usées et les boues d'épuration. La deuxième section illustre les propriétés d'adsorption de divers microbiochars. En particulier, l'élimination du DCF a été étudiée par adsorption sur divers microbiochars et des microbiochar fonctionnalisés à l'acide organique ont été testés pour une élimination améliorée du DCF. La troisième section étudie la production rentable d'enzyme laccase ligninolytique pour la dégradation du DCF. De plus, des méthodes d'adsorption ainsi que d'immobilisation covalente de la laccase sur des microbiochars ont été étudiées dans cette section. Enfin, la quatrième section traite le "développement d'un microsystème imprégné de biochar-laccase" pour l'adsorption continue ainsi que la dégradation du DCF. **L'hybride " microsystème imprégné de Biochar-Enzyme " en encapsulant des enzymes sur le biochar sera prometteur comme une approche efficace, durable, verte ainsi rentable pour le traitement de DCF, thème clé de cette thèse.**

PARTIE 2: REVUE DE LITTÉRATURE

2.1 Les contaminants préoccupants - composés pharmaceutiquement actifs (PhACs)

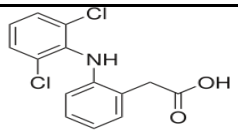
Les contaminants émergents comprennent : les produits pharmaceutiques et de soins personnels (PPCPs), les herbicides, les pesticides et les perturbateurs endocriniens, etc. Ces produits chimiques sont souvent détectés, comme éléments traces (micropolluants), dans divers contaminants environnementaux. Pour beaucoup d'entre eux, le risque potentiel pour l'environnement et la santé humaine est inconnu. Les composés pharmaceutiquement actifs (PhACs) représentent une classe importante dans les produits pharmaceutiques qui par une façon ou d'une autre se diffusent dans l'environnement en tant que composé parent ou sous forme de métabolites pharmacologiquement actifs (Halling-Sørensen et al., 1998). La consommation mondiale des composés actifs est estimée à 100.000 tonnes ou plus par an (Kummerer, 2004). La

mise au point des médicaments a pour objectif d'apporter un effet biologique bénéfique sur l'organisme auquel ils sont administrés. Néanmoins, un effet biologique indésirable peut s'exercé par ces composés sur l'environnement (Halling-Sørensen et al., 1998). La présence globale de composés pharmaceutiques et de PhACs dans l'environnement aquatique est une problématique émergente dont les conséquences demeurent inconnues.

2.2 Les anti-inflammatoires non stéroïdiens (NSAIDs) - Diclofenac (DCF)

Les médicaments anti-inflammatoires non stéroïdiens (NSAIDs) sont des PhACs largement utilisés dans le monde entier. Ils sont détectés dans différents compartiments de l'environnement à des concentrations allant de ng L^{-1} à faible mg L^{-1} (Halling-Sørensen et al., 1998; Khetan and Collins, 2007). De plus, les NSAIDs sont des médicaments <<*over-the-counter* >>(OTC) dans la plupart des pays ce qui contribue à l'augmentation de la disponibilité et de la consommation. Le Diclofénac (DCF), reconnu comme l'«antidouleur le plus populaire du monde», est aussi le NSAIDs le plus couramment utilisé. Sa part de marché correspond presque à celle des trois autres médicaments les plus répandus (ibuprofène, l'acide méfénamique, le naproxène) combinées (McGettigan and Henry, 2013). Le nom de diclofenac est dérivé de sa nomenclature: 2-(2,6-dichloranilino) acide phénylacétique. Il a été découvert, en 1973, par la société pharmaceutique suisse Ciba-Geigy (maintenant fusionné à Novartis). Le DCF est couramment utilisé pour réduire l'inflammation et pour soulager la douleur dans des cas, tels que l'arthrite ou les blessures graves. Il fonctionne aussi comme anti-uricosurique et analgésique.

Tableau 1: Les propriétés physico-chimiques et les propriétés pharmacologiques de diclofénac

Structure	
Formule moléculaire et poids moléculaire	$C_{14}H_{11}Cl_2NO_2$; 296,16 g mol^{-1}
N ° CAS	15307-86-5 15307-79-6 (sel disodique)
Solubilité dans l'eau	2,37 mg L^{-1} (25 ° C)
Constante de la loi de Henry	4,79 × 10^{-7} Pa m^3 mol^{-1} (25 ° C)
Les points de fusion et d'ébullition	283-285 °C et 412 °C à 760 mm Hg (prédite), respectivement
pKa	4.15
Log K_{ow} (logarithme du coefficient de partage octanol-eau)	4.51

Référence : syrres.com, www.sigmaaldrich.com, www.drugbank.ca, www.scbt.com

Le médicament présente une mauvaise solubilité dans l'eau dans son état libre. En général, il est utilisé sous forme de sels de sodium ou de potassium pour améliorer la solubilité et l'absorption. En outre, la faible constante de Henry (tableau 1) est un indicateur de la mauvaise élimination du médicament des eaux usées.

2.2.1 La consommation mondiale et de la législation

Pour diverses raisons, il est pratiquement impossible de calculer la consommation globale exacte de diclofénac. En autres, l'utilisation des différents noms commerciaux pour le DCF, les diverses applications (p. ex. la médecine humaine et vétérinaire) et le fait qu'il soit un médicament en vente libre. Cependant, Zhang et al. (2008) ont estimé, sur une base annuelle, qu'environ 940 tonnes de DCF sont consommées dans le monde à partir des données de santé de l'Intercontinental Marketing Services (IMS). Dans 76 pays, représentant environ 96% du marché pharmaceutique mondial de diclofénac, environ 877 tonnes de diclofénac ont été vendues en 2007 (Zhang et al., 2008).Dans un rapport de « Fierce Pharma » en 2012, le diclofénac a été répertorié comme la 12^{e} molécule générique best-seller à l'échelle mondiale. Le total des ventes de diclofenac en 2011 a été estimé à 1,61 milliard de dollars avec une charge des ventes annuelles de 15,5% (Palmer, 2012). De plus, une étude récente a estimé la consommation mondiale du DCF à 1443 ± 58 tonnes (Acuña et al., 2015).

La présence du DCF dans l'environnement a été reconnue récemment comme un risque pour la santé des organismes terrestres. Il est extrêmement toxique pour les vautours, même si elles ne le consomment pas directement, c'est son utilisation pour le bétail qui a anéanti et menacé les populations de vautours en Inde, au Pakistan et au Népal puisqu'ils consomment les carcasses de bétail. L'Inde a été le premier pays à imposer des réglementations sur la consommation du DCF. En 2006, la fabrication et l'utilisation vétérinaire du DCF ont été interdites en Inde (The Drug controller general, 2006). De plus, en 2008, l'Inde a imposé des restrictions supplémentaires sur l'utilisation du diclofénac pour les animaux. Une contravention est passible d'une peine d'emprisonnement. En cette même année, le Népal et le Pakistan ont interdit la drogue à usage vétérinaire suivie par le Bangladesh en 2010 (K.Venkateshwarlu, 2011). Pour l'usage vétérinaire, le diclofénac ne dispose pas d'une autorisation de commercialisation centralisée de l'Agence européenne des médicaments (AEM) et il est autorisé de façon indépendante dans chaque État membre. En outre, l'AEM a fixé une limite maximale de résidus pour le DCF dans les espèces bovine et porcine. Depuis 2013, la

production commerciale du DCF a commencé en Espagne et en Italie et l'exportation s'est effectuée vers les autres pays de l'Union européenne. Une coalition de célèbres organisations, dont la Fondation pour la conservation des Vautours, La Société royale pour la protection des oiseaux, BirdLife Europe, et le Groupe de spécialistes de l'IUCN Vautour, font campagne pour mettre en place un imposé qui interdit, à l'échelle du continent européen et suivant les leçons de l'expérience indienne, le diclofénac vétérinaire (BirdLife, 2013; Tavares, 2014a; Tavares, 2014b). Récemment, le Diclofénac a été ajouté aux normes de qualité environnementale (NQE) de l'Europe. Selon le document de la Communauté européenne (COM (2011) 876), la valeur moyenne annuelle de la NQE (évaluation des normes de qualité) pour le DCF était de 0,1 µg L^{-1}. Toutefois, ce document a été modifié et le DCF est mis sur la liste de surveillance jusqu'à prochaine révision (Johnson et al., 2013). En 2013, le DCF a été inclus dans la liste de surveillance de la « directive-cadre sur l'eau » afin de recueillir suffisamment de données de surveillance dans le but de la détermination des mesures de réduction des risques. Selon le document NQE proposé, les concentrations maximales admissibles sont 0,1 µg L^{-1} dans les eaux douces et 0,01 µg L^{-1} dans les eaux marines. Le Royaume-Uni (RU) a placé le DCF dans « la liste des substances prioritaires » ce qui oblige les industries de l'eau à mettre en place des technologies de recherche pour enlever le DCF des eaux usées.

2.3. Devenir du DCF dans l'environnement

2.3.1 Devenir dans l'environnement

Bien qu'ils aient, à la base, une influence positive sur la santé humaine ou animale, les produits pharmaceutiques ont souvent des effets néfastes sur l'environnement. Lorsqu'ils rentrent en contact avec ce dernier, ils peuvent affecter les mêmes voies biologiques chez les animaux ayant des organes cibles, des tissus, des cellules ou biomolécules identiques ou similaires (Fent et al., 2006). Des études ont démontré les effets négatifs potentiels de diclofénac dans l'environnement (Cleuvers, 2004; Gros et al., 2010; Kunkel and Radke, 2012; Oaks et al., 2004). Par conséquent, il

est important de comprendre l'origine et le devenir des produits pharmaceutiques, et du diclofénac en particulier, dans l'environnement pour concevoir des mesures appropriées correctives de pollution. Étant un polluant d'origine anthropique, la source de diclofénac est l'industrie pharmaceutique, et il est utilisé à la fois pour des fins humaine et vétérinaire. Suite à ces utilisations, le DCF se retrouve dans les stations d'épuration des eaux usées ou dans les décharges sous forme de DCF ou un de ses métabolites. De même, le procédé de traitement conventionnel du DCF dans les stations d'épuration des eaux usées est inefficace (Fatta-Kassinos et al., 2011; Zorita et al., 2009) et donc le DCF peut se retrouver dans l'eau de surface et la possibilité pour sa percolation aux sources d'eau potable ne peut pas être annulée. En outre, les probabilités de percolation du DCF, des décharges jusqu'à l'eau de surface, sont assez élevées. L'effet potentiel néfaste du DCF dans le milieu aquatique a été révélé dans de nombreuses études (Cleuvers, 2003; Fent et al., 2006; Jones et al., 2002; Lee et al., 2011).

2.3.2 Présence dans les milieux naturels

Le DCF a été largement détecté dans divers milieux aquatiques. Dans les eaux de surface, il a été détecté en nano grammes par litre alors que, dans les eaux usées, la concentration était aussi élevée que des microgrammes par litre. La diminution de la concentration par des processus naturels, tels que la rétention des sols, la biodégradation et la photo-transformation et également par des procédés physico-chimiques dans les usines de traitement des eaux usées. Dans les eaux de surface, la contamination par le DCF a eu lieu dans les rivières, les estuaires et les lacs (Buser et al., 1998; Kim et al., 2007; Metcalfe et al., 2003; Öllers et al., 2001). En outre, il y a quelques rapports sur sa détection dans les eaux souterraines et l'eau potable (Benotti et al., 2008; Rabiet et al., 2006). La plupart des cas de détection concernent les pays de l'UE; toutefois, cela ne signifie pas que le DCF était présent dans les pays européens. En Asie, les données disponibles étaient insuffisantes pour prédire les concentrations dans l'environnement, car il n'y avait pas de données annuelles systématiques. Par rapport à l'Europe, la consommation du DCF en Amérique du Nord

était plus faible. Cependant, il n'y a que quelques cas de détection du DCF dans l'environnement qui soient signalés (Metcalfe et al., 2003; Sosiak and Hebben, 2005).

Plus de 4900 ng L-1 des concentrations en Diclofenac a été détecté au Pakistan dans l'eau des rivières (Scheurell et al., 2009) et cela peut être dû à un manque de traitement approprié des eaux usées dans les pays asiatiques. Les masses d'eau allemandes ont également été fortement polluées par le DCF et des concentrations aussi élevées que 1 030 ng L-1 ont été détectées dans l'eau des rivières (Heberer, 2002a). De plus, les résidus de DCF ont été détectés dans presque tous les pays de l'UE (Hernando et al., 2006; Loos et al., 2010). Aux États-Unis et en Allemagne, le DCF a même été détecté dans l'eau potable ce qui a mérité de l'attention (Benotti et al., 2008; Heberer, 2002b).

Il y a quelques cas signalés de détection du DCF dans le sol. Le Diclofénac pourrait atteindre les terres agricoles par l'application des boues d'épuration municipales en tant que source de nutriments dans le sol ou par les eaux usées et il a été détecté dans la province canadienne de l'Ontario (Al-Rajab et al., 2010). Des études sur le coefficient de sorption de diclofénac ont prouvé que la sorption, même dans les sédiments sableux, était pertinente et donc le diclofénac était moins mobile dans les eaux souterraines (Scheytt et al., 2005). D'autre part, des études d'Israël ont rapporté que le DCF montre une mobilité plus lente dans les sols agricoles riches en matières organiques et une plus grande mobilité dans les colonnes d'eau douce ce qui a provoqué son lessivage jusqu'aux eaux souterraines et, en fin de compte, jusqu'à l'eau potable après les événements de pluie (Chefetz et al., 2008; Drillia et al., 2005). Peu d'autres études ont également souligné la même possibilité (Chefetz et al., 2008; Xu et al., 2009).

2.4 Préoccupations concernant la toxicité du DCF

De nombreuses études ont été menées dans le monde entier pour évaluer la toxicité des DCF dans les organismes aquatiques. L'une des méthodes largement utilisées et très standardisées pour

mesurer la toxicité était les tests d'immobilisation aiguë. Ce sont eux (Ferrari et al., 2003) qui ont mené l'une des premières études sur les effets toxiques du DCF. Cette étude a été sur les bactéries, les algues, les microcrustacés, et les poissons, et a montré des effets relativement moins toxiques, même à des concentrations environnementales. Au contraire, des études ultérieures ont révélé les impacts potentiels de diclofénac sur l'environnement. Selon les études d'évaluation des risques, le risque écologique potentiel du diclofénac dans les eaux de surface était plus élevé(Hernando et al., 2006). Cleuvers (2004) a mené des études d'écotoxicité en utilisant des tests sur Daphnie aiguë et les algues et a révélé que le DCF était potentiellement dangereux pour les organismes aquatiques. Dans la même étude, Cleuvers (2004) révèle également que, sous champs ou sous des concentrations environnementales, les effets indésirables ont été minimes ou négligeables et qu'un mélange de produits pharmaceutiques peut être considéré comme toxique, même à des concentrations plus faibles. Dans les crustacés (*Daphnia magna* sp.), des concentrations aiguës de DCF, telles que des mg L^{-1}, induisent des taux de mortalité élevés. À la base de différentes études sur l'exposition de 48h, la présence de DCF produit une mortalité élevée et les valeurs de CE_{50} ont été signalées à 22,4 mg L^{-1}et 39,9 mg L^{-1}(Ferrari et al., 2003; Haap et al., 2008). En outre, pour *Ceriodaphnia dubia* sp., la mortalité a été observée et la CE_{50} était de 22,7 mg L^{-1} (Ferrari et al., 2003). Au contraire(Lee et al., 2011) ont rapporté 3 fois des valeurs élevées pour les tests de 48h EC_{50} pour la même espèce. Étonnamment, des études de 2007 réalisées au Canada ont signalé que le DCF était un risque majeur, même à des concentrations prévues dans l'environnement (Lawrence et al., 2007). Ces études sur les communautés biofilm de rivière ont révélé des impacts significatifs des DCFs sur la structure des communautés et leurs fonctions sous mêmes concentrations plus faibles que 100 ng L^{-1}.

Le DCF était connu pour exercer des effets mortels en endommageant les tissus rénaux et gastro-intestinaux dans plusieurs vertébrés, tels que les poissons. Dans une étude d'évaluation de l'exposition (Letzel et al., 2009) ont constaté que, à des concentrations pertinentes pour l'environnement, telles que nanogrammes par litre, le DCF peut entraîner des effets néfastes

chroniques sur les populations de poissons. Chez les poissons, medaka japonaise (*Oryzias latipes)*, le DCF affecte négativement la croissance en phase d'oeufs et entraîne une réduction significative des taux d'éclosion et un retard dans l'éclosion (Lee et al., 2011). Dans une étude sur le poisson-zèbre (Hallare et al., 2004) ont observé les mêmes résultats. L'éclosion a été retardée lorsque l'éclosion des embryons a été exposée à 2000 µg L^{-1}. Dans la truite brune, le DCF n'a pas été complètement éliminé par métabolisme du premier passage, mais une partie importante de DCF est entrée dans la circulation entéro-hépatique. La résultante prolongeait la disponibilité du DCF dans l'organisme qui favorise son accumulation (Hoeger et al., 2008). Pour la même espèce, un des dommages importants sur les branchies, le foie et le rein a été observé à 50 µg L^{-1} (Hoeger et al., 2005). Pour la truite arc-en-ciel, même à des concentrations observées pour l'environnement, le DCF a gêné le fonctionnement biochimique et a conduit à des lésions tissulaires (Mehinto et al., 2010; Schwaiger et al., 2004). Le DCF peut s'accumuler dans le foie, les reins et les branchies des tissus musculaires de la truite arc-en-ciel et il peut provoquer des altérations cytologiques même à 1 µg L^{-1} (Schwaiger et al., 2004; Triebskorn et al., 2004). Les moules sont affectées par le DCF à des concentrations qui sont répandues dans l'environnement. À des niveaux de concentration de nanogrammes par litres, le DCF induite significativement à la peroxydation lipidique (POL) indiquant une lésion tissulaire dans les moules (Schmidt et al., 2011). Une étude relativement récente de (Gonzalez-Rey and Bebianno, 2014) a démontré qu'à 250 ng L^{-1}, ce qui est très proche des concentrations de certains cours d'eau allemandes, le DCF induit à des réponses de biomarqueurs spécifiques conduisant à l'endommagement des tissus. Il a également une incidence négative sur le métabolisme et la croissance des moules bleues qui sont communs dans la mer baltique (Ericson et al., 2010).

C'est l'effondrement soudain de vautour en raison de la consommation de carcasses contenant des résidus de DCF qui a été le premier cas largement signalé sur les produits pharmaceutiques causant des dommages écologiques, et qui menace d'extinction plusieurs espèces de vautours (Oaks et al., 2004; Taggart et al., 2007). Le DCF a été la principale cause de l'effondrement de la

population de trois espèces de vautours Gyps (*Gyps bengalensis, Gyps indicus, Gyps tenuirostris*). Ces espèces ont été sévèrement touchées. En effet, une réduction de 98% dans le sous-continent indien a été constatée et il a été inclus pour les espèces, listées par l'IUCN, « *en danger critique* » (Das et al., 2010).

2.5 Métabolites DCF et produits de transformation

Après sa consommation par les animaux, le DCF est principalement dégradé en dérivés hydroxylés. Il est dégradé assez facilement pour des produits de transformation dans l'environnement. Le principal processus naturel de dégradation est la phototransformation par la lumière du soleil. Le DCF est l'un des résidus pharmaceutiques le mieux étudiés dans l'environnement; cependant, des études sur l'apparition et la toxicité de ses métabolites dans l'environnement sont encore mal comprises.

Dans le corps humain, le dérivé du DCF est trouvé dans l'urine et le plasma. Les dérivés hydroxylés et méthoxylés de DCF sont présents sous leurs formes libres ainsi que des formes glucuronide conjuguées. Dans une étude antérieure, Stierlin et al. identifiaient les métabolites du DCF dans le corps humain et le métabolite principal a été le 4'-hydroxydiclofenac (2-[2,6-dichloro-4 hydroxyphénylamino] d'acide phenylethanoic) (30%) et d'autres métabolites majeurs sont le 5'-hydroxydiclofenac (2-[2,6-dichlorophénylamino]-5-hydroxyphe acide nylethanoique) (10%), 3'-hydroxydiclofenac (2-[2,6-dichloro-3-hydro xyphenylamino] acide phenylethanoique) et 4',5-dihydroxydiclofenac (2-[2,6-dichloro-4-hydroxyphénylamino]-5-acide hydroxyphenylethanoique) (15%) (Boettcher et al., 1991; Stierlin et al., 1979)**.** Récemment, certains métabolites mineurs ont également été identifiés dans le corps humain (Stulten et al., 2008a). En plus des dérivés d'hydroxyle des DCFs, l'acyle glucuronide et l'acyle glucuronide hydroxyle ont également été trouvés chez les souris et les poissons (Kallio et al., 2010; Naisbitt et al., 2007). La grande présence dans métabolites humains du diclofénac dans l'eau et sa similitude structurelle au diclofénac est un sujet de préoccupation pour des raisons de toxicité et doit être étudiée de façon plus poussée.

Le DCF est facilement dégradé dans la lumière du soleil. La demi-vie était inférieure à 3,3 h (Schmitt-Jansen et al., 2007). Il suit une cinétique du premier ordre au cours de la photodégradation et a été détecté dans le cycle de l'eau.(Qin and Yang, 2012). La photodégradation a été identifiée comme le principal processus de la dégradation des DCF dans les lacs et il a été estimé qu'environ 90% des DCF ont été éliminés par ce processus (Buser et al., 1998). De nombreux produits issus de la phototransformation du DCF ont été identifiés par divers chercheurs (Agüera et al., 2005; Moore et al., 1990; Qin and Yang, 2012). Le procédé le plus important de la phototransformation du DCF a été identifié comme étant la photocyclisation au carbazole monohalogéné correspondant (Eriksson et al., 2010). La plupart des produits de décomposition photochimiques se sont produits comme deux sous-structures principales: 2-chloro- et 2,6-dichlorodiphenylamine et également des dérivés de 8-hydroxy et 8-chlorocarbazole dérivé (Agüera et al., 2005; Moore et al., 1990).

Le diclofénac ainsi que ses métabolites entrent dans le monde de l'environnement aqueux. Il a été rapporté que certains des métabolites du DCF sont encore plus toxiques que le DCF. Certains des produits de phototransformation ont montré une augmentation de six fois la toxicité dans les tests de reproduction des algales (Schmitt-Jansen et al., 2007; Schulze et al., 2010). Compte tenu des effets toxiques du diclofénac sur plusieurs organismes aquatiques, il semble très probable que les métabolites ont également induit des réactions indésirables sur d'autres organismes et nécessitent une stricte surveillance au cours des expériences de surveillance toxicologique et environnementale des médicaments(Stulten et al., 2008b).

2.6 Scénario de traitement actuel pour l’enlèvement du DCF de l'environnement

2.6.1 Le traitement conventionnel de DCF dans les stations d'épuration des eaux usées

Le système de traitement classique, dans les stations de traitement des eaux usées, présente une efficacité entre modérée et élevée de la élimination de DCF (tableau 2). Le DCF est modérément persistant dans l'environnement. Dans les études examinées, la suppression maximale obtenue est d'environ 93% par adsorption sur charbon actif suivi par l’ozonation (Beltran et al., 2009). Le

traitement primaire a également été efficace avec des agents coagulants et floculants, tels que $FeCl_3$, $Al_2(SO_4)_3$, mais n'a pas capable d'éliminer complètement le médicament des eaux usées (Carballa et al., 2005)**.** Le processus de boues activées classiques a montré une meilleure efficacité par rapport au BRM. En moyenne, la suppression de 30-70% peut être obtenue par les méthodes d'élimination existantes (Vieno and Sillanpää, 2014; Zhang et al., 2008). Une vue d'ensemble des méthodes de traitement est donnée dans le tableau 2. L'efficacité d'élimination dépend principalement des méthodes de traitement. Par exemple, le procédé des boues activées classiques assure l'élimination de 75% (Kimura et al., 2005), mais la plupart du temps ce retrait a été réalisé par le transfert de masse. Par conséquent, l'élimination efficace ou une dégradation du médicament est minime dans le système de traitement classique des eaux usées. Plusieurs autres options de traitement qui sont basées sur le processus de sorption ont été récemment proposées par divers chercheurs (Sotelo et al., 2014; Sotelo et al., 2012; Suriyanon et al., 2013). Même si divers matériaux adsorbants naturels et synthétiques, tels que le charbon actif, le biochar, adsorbants à base de polymère à base de silice, présentent une excellente efficacité d'élimination du DCF, dans l'ensemble, ces processus ne peuvent pas être considérés comme des méthodes durables; parce que ces processus ne suppriment pas complètement le DCF de l'environnement; le DCF lié à l'eau /aux eaux usées est accumulée sur l'adsorbant. Un autre procédé, le plus largement utilisé pour le traitement de DCF, est basé sur des procédés d'oxydation avancée tels que l'ozonation (Beltran et al., 2009); cependant, ces méthodes ont aussi reçu des retombées. Ces processus peuvent créer sous-produits à effet indésirable et toxique. Néanmoins, quelques développements récents sont prometteurs et durables. Des études ont démontré l'utilisation efficace des enzymes pour la dégradation complète du DCF de l'eau (Marco-Urrea et al., 2010) qui ne créera pas des sous-produits nocifs.

Tableau 2: Devenir du Diclofénac dans les usines de traitement des eaux usées

Méthode de traitement majeure	Les conditions du procédé et d'autres procédés de traitement	% de Retrait	Commentaires	Référence
BRM submergé	flux de membrane 0,4 m^3/ml/j TRH 9h SSLM de 10 000 mg L^{-1}	40	--------	(Kimura et al., 2005)
Boues activées conventionnelles	TRH 13h SSLM de 1700 mg L^{-1}	75	--------	(Kimura et al., 2005)
Traitement biologique conventionnel des eaux usées	--------	65	résultat moyen d'étude sur 5 stations de traitement biologique en Espagne	(Gomez et al., 2007)
Le traitement primaire (Coagulation et la flottation)	Coagulation-$FeCl_3$,$Al_2(SO_4)_3$ PAX Flotation- 12 ° C -25 ° C	40-70 20-40	--------	(Carballa et al., 2005)
Traitement classique de boue activée (CAS)	Pré-dénitrification anoxique et la précipitation du phosphate avec du chlorure ferrique en tant que traitement tertiaire	65	15% à 65% de l'élimination totale par adsorption sur les boues	(Larsson et al., 2013)

Tableau 2: Devenir du Diclofénac dans les usines de traitement des eaux usées (suite)

Méthode de traitement majeure	Les conditions du procédé et d'autres procédés de traitement			% de Retrait	Commentaires	Référence
Traitement biologique classique des eaux usées	Traitement par biofiltration des eaux usées et composé de 30 filtres biologiques			75	Certains % de retrait ont été produits par l'adsorption sur les boues	(Lonappan et al., 2016)
Processus de boues activées	--------			25	--------	(Martín et al., 2012)
BRM	TRS (j) - 10-55 TRH (j) -0,5-4			<50	--------	(Clara et al., 2005)
Traitement biologique conventionnel des eaux usées	STEP	TRS	TRH		Étude sur 2 STEPs. L'accumulation sur les boues était mineure.	(Samaras et al., 2013)
	1	8 j	9 h	75		
	2	18 j	23 h	39		

Les abréviations - BRM: bioréacteur à membrane, TRH: temps de rétention hydraulique, TRS: Temps de rétention de la sédimentation, SSLM: solides en suspension dans la liqueur mixte, STEP: Station d'Épuration des eaux usées.

2.6.2 Enlèvement par adsorption- une introduction à l'adsorption biochar

Au cours des dernières décennies, l'adsorption est apparue comme une méthode de traitement efficace pour l'élimination de divers contaminants organiques émergents, tels que les produits pharmaceutiques (Nam et al., 2014; Westerhoff et al., 2005) et des adsorbants classiques, tels que le charbon actif ont été largement étudiés pour l'élimination de CECs (Westerhoff et al., 2005). Les propriétés physico-chimiques des adsorbants et des adsorbats (p. ex. l'hydrophobie des groupes fonctionnels chimiques), la taille des pores, la surface externe et la composition chimique ainsi que d'autres conditions de réaction (p. ex. le pH et la température) déterminent l'efficacité d'élimination (Nam et al., 2014; Nielsen et al., 2014). Par conséquent, tous les adsorbants même après l'activation de surface ne peuvent pas devenir un excellent adsorbant pour tous les contaminants en général. Par exemple, la plupart des charbons actifs vont exclure des molécules grosses (> 3000 Da), en raison de sa petite taille de pores (< 2 nm) (Kilduff et al., 1996). De plus, l'activation est une étape supplémentaire et coûteuse. Ainsi, les adsorbants économiques et respectueux de la nature doivent être identifiés spécifiquement pour le composé à éliminer.

En tant que solution de gestion des déchets, le biochar de matière carbonée a exposé son potentiel dans le développement durable et a démontré son utilité en tant que produit de valeur ajoutée pour plusieurs applications environnementales (Glaser et al., 2009; Lee et al., 2010; Woolf et al., 2010). La production de matériaux durables et les pratiques de gestion avancée pour les déchets, tels que la production de biochar, gagnent l'attention dans le monde entier. Le biochar est un matériel négatif en carbone (Glaser et al., 2009) et par conséquent, sa production et sa consommation sont attrayantes vers un avenir durable. En plus d'être une voie pratique et rentable pour l'élimination des résidus organique. De plus, il trouve même ses applications dans l'atténuation du changement climatique par la séquestration du carbone, les applications simultanées dans la production d'énergie et l'augmentation du rendement des cultures (Woolf et al., 2010). La capacité et les mécanismes d'adsorption varient de façon significative, avec la charge en matière première et la méthode de production, suivant les changements dans les propriétés chimiques et de la chimie de

surface. Le biochar joue le rôle d'un excellent adsorbant pour plusieurs contaminants de l'environnement grâce à la structure poreuse et la chimique de surface. Plusieurs facteurs tels que le pH, la température, la surface spécifique, la porosité, la composition chimique, des charges de surface et les groupes fonctionnels présents sur la surface auront une influence directe sur la capacité d'adsorption ainsi que sur le mécanisme et le processus d'adsorption. Une bonne compréhension de l'interaction entre adsorbat et adsorbant est nécessaire pour concevoir des systèmes d'adsorption efficaces pour l'élimination des contaminants. En raison de l'aromaticité et l'hydrophobie, le biochar est un excellent agent de sorption pour un certain nombre de composés organiques hydrophobes et de composés inorganiques (Fang et al., 2014). Étant une option respectueuse de l'environnement pour la gestion des déchets, le biochar est un adsorbant prometteur pour l'élimination des micropolluants. Ce potentiel vient en raison de ses propriétés supérieures, y compris une structure très condensée et la densité superficielle des groupes fonctionnels (Jung et al., 2015). Par conséquent, il peut être utilisé comme un excellent adsorbant à la fois pour les polluants cationiques et anioniques. Le potentiel d'adsorption peut être amélioré en modifiant la surface du biocharbon et le traitement à l'acide organique peut être un outil efficace pour modifier la surface. Récemment, l'adsorption du DCF a été étudiée par divers adsorbants, tels que les nanotubes de carbone à parois multiples (MWCNT) (Czech and Oleszczuk, 2016), l'oxyde de graphène (Nam et al., 2015) et le charbon actif (Jung et al., 2013). Cependant, le potentiel d'adsorption du biochar pour « les micropolluants » tels que le DCF n'a pas été étudié. En raison de ses propriétés de surface spécifique; qui peuvent être attribuées en grande partie à la sélection des matières premières initiales et la méthode de production; les différents types de biochars peuvent avoir un excellent potentiel d'adsorption pour le DCF.

2.6.3 La dégradation des procédés PhACs-enzymatiques

Les enzymes ligninolytiques, les laccases en particulier, sont connus pour transformer les contaminants de l'environnement (Keum and Li, 2004; Youn et al., 1995). Ils présentent également une excellente capacité de dégradation des contaminants émergents, tels que les produits

pharmaceutiques (Gassara et al., 2013b).Laccases (1.10.3.2,p-diphénol: oxydoréductases dioxygène), les manganèse peroxydases et les lignine peroxydases sont connus comme des enzymes ligninolytiques ou enzyme de modification de la lignine (EML). De toutes les enzymes oxydatives, la laccases est celle qui peut être appliquées à la plus large gamme de substrats, surtout pour la bioremédiation (Majeau et al., 2010). Laccases sont des glycoprotéines (monomère, dimères ou tétramères) contenant quatre atomes de cuivre par monomère situé au niveau du site catalytique. Avec les atomes de cuivre; il peut y avoir trois types: type 1, type 2 ou type 3. Le type 1 attire la plupart des études puisqu'il est responsable de l'oxydation du substrat, et il fournit en outre une couleur bleue à l'enzyme (Majeau et al., 2010). L'important mécanisme impliqué dans l'action de la laccase dans la dégradation des contaminants est l'oxydation par l'oxygène moléculaire. À l'aide de mécanismes impliquant des radicaux, l'oxygène moléculaire, provenant de la laccase, oxyde les contaminants qui contiennent des donneurs d'hydrogène. En outre, les radicaux produits peuvent subir des réactions catalysées par la laccase et/ou des réactions non enzymatiques, tels que la polymérisation, l'hydratation ou l'abstraction d'hydrogène (Majeau et al., 2010), ce qui contribuera à faciliter le processus de dégradation. L'un des plus grands avantages de la laccase sur plusieurs autres enzymes est sa stabilité. La stabilité de l'enzyme peut varier avec le micro-organisme qui est utilisé pour la production de laccase. Par exemple, la demi-vie de la laccase purifiée à 50 °C peut varier, en général, de quelques minutes pour l'enzyme produite par *Botrytis cinerea (Slomczynski et al., 1995)* à plusieurs jours pour l'enzyme produite à partir de *Trametes* sp. (Smirnov et al.). En outre, les exigences pour la catalyse par la laccase sont assez simples en raison de sa stabilité apparente et le manque d'inhibition. De récentes études ont rapporté l'utilisation d'une enzyme ligninolytique, en particulier la laccase, pour la dégradation de plusieurs CEC y compris PhACs (Cruz-Morató et al., 2013; Rodríguez-Rodríguez et al., 2011; Tran et al., 2013; Wen et al., 2010).

Toutefois, pour des fins d'assainissement, de grandes quantités de l'enzyme sont nécessaires et pour les méthodes classiques, le coût de production peut être très élevé (Majeau et al., 2010). En vue de réduire le coût de production de la laccase, l'utilisation des déchets provenant de diverses

sources, tel que l'agriculture et les industries de transformation alimentaire peuvent être utilisées comme substrat pour le micro-organisme (Songulashvili et al., 2006). Récemment, les déchets de l'industrie de la pomme (de la purée de pommes) ont été identifiés comme un substrat approprié pour produire différentes enzymes (Gassara et al., 2013a; Gassara et al., 2011). En 2011, au Canada, la production totale de pommes commercialisées était de 390,362 tonnes métriques et dont plus de 25% provenaient de la province de Québec seulement (Statistics, 2012). Par conséquent, le Canada est l'un des plus grands producteurs de pommes. Il génère des milliers de tonnes de déchets chaque année compte tenu du fait que l'industrie de transformation de jus de pomme génère 25% à 30% de purée de pommes (Ajila et al., 2015; Dhillon et al., 2011). À l'heure actuelle, une grande partie de la pulpe de pomme produite à partir de l'industrie du jus de fruits a été utilisée comme aliment pour animaux ou d'engrais au sol. En fait, l'option d'élimination de la purée de pommes via l'alimentation des animaux est assez limitée puisqu'ils présentent une faible digestibilité. En effet, ce type de déchet présente une forte proportion de lignine/cellulose et de faibles quantités de nutriments comme les protéines, les vitamines et autres minéraux essentiels pour la croissance des animaux (Ajila et al., 2015; Rumsey, 1978). Par conséquent, l'industrie de la pomme peut être considérée comme un candidat potentiel pour fournir le substrat nécessaire pour la production des EMLs.

2.6.4 Microsystèmes imprégné d'enzyme du Biochar (BEMS) pour le traitement du DCF

La réduction de la taille du biochar peut être une approche efficace pour l'élimination des contaminants par le processus d'adsorption puisque la réduction de la taille augmentera la superficie totale. En général, le biochar, préparé via le procédé de pyrolyse, est de grandes dimensions, soit de l'ordre de micromètres ou de millimètres en fonction de la matière source et du procédé de production. Il est, par la suite, utilisé comme obtenu par le procédé (Demirbas, 2004; Lei et al., 2009). L'effort de classer les microparticules selon la taille est rare, sauf pour les nanoparticules (Kambo and Dutta, 2015; Yan et al., 2013). La plupart des études d'adsorption précédentes se concentraient sur le biochar (Ahmad et al, 2012; Cantrell et al, 2012; Gai et al, 2014.). Ces études,

qui visent l'élimination des contaminants organiques, ne sont pas en mesure de fournir une excellente efficacité d'élimination. Peu d'études récentes ont évalué l'efficacité des nanoparticules de biochar en tant qu'un adsorbant autonome pour les contaminants de l'eau et du sol en combinaison avec d'autres nanomatériaux (Saxena et al, 2014; Yan et al, 2013), et aussi en tant qu'un catalyseur au même temps que d'autres matériaux (Yan et al., 2013). Ces études ont montré l'excellente capacité pour l'élimination des contaminants organiques. Par conséquent, il peut être prévu que le nanobiochar présentera une excellente capacité d'adsorption, en comparaison avec le biochar brut ou même le microbiochar, en raison de sa plus grande surface de contact, même si la préparation de nanoparticule est un processus coûteux. D'autre part, avec des microparticules, une augmentation du potentiel d'adsorption peut être attendue en comparaison avec le biochar obtenu sous la forme brute sans implication importante des coûts. De plus, la synthèse et la caractérisation de microparticules de biochar pour l'adsorption restent à étudier et des recherches supplémentaires sont nécessaires dans ce domaine. D'une manière générale, les nanoparticules agglomèrent sous forme de liquide ce qui est un facteur important. Cela permettra de réduire la capacité d'adsorption puisque l'agglomération va réduire la surface effective des nanoparticules. En cas de microparticules de biochar, la possibilité d'agglomération peut être exclue en comparaison avec des nanoparticules. En outre, en raison de ses uniques propriétés environnementales, des nanoparticules de carbone sont toxiques pour de nombreux organismes (Brar et al., 2010; Firme Iii and Bandaru, 2010). Par conséquent, l'élimination des nanoparticules après l'utilisation peut être un problème majeur. Tant que le biochar est considéré comme une option pour la gestion des déchets et la production de « nanoparticules toxiques », cela ne constitue pas une méthode efficace pour la réduction des déchets et d'autre part les microparticules de biochar ne sont pas encore révélées être toxiques. Par conséquent, l'option d'utilisation des microparticules de biochar pour l'adsorption et la gestion des déchets peut être considérée.

Les enzymes telles que les laccases sont utilisées à des fins d'assainissement. Cependant, le fait que ces enzymes libres ne soient pas réutilisables et soient sensibles aux agents dénaturants

présente des inconvénients majeurs. Par conséquent, leurs utilisations sans aucun support nécessitent beaucoup plus d'activité enzymatique et ne peuvent pas être réutilisées, car l'enzyme ne peut pas être récupérée. En outre, la plupart des enzymes qui ont la forme libre sont beaucoup plus sensibles au pH, à la température et aux agents dénaturants que ceux sous forme immobilisée. L'immobilisation d'enzymes va augmenter sa stabilité et va fournir une protection supplémentaire par rapport à la dénaturation par un ensemble de co-solvants organiques (D'Annibale et al., 2000). Elle fournit, en plus, une excellente efficacité catalytique même après plusieurs cycles de réaction (Palmieri et al., 2005) et aide à séparer les produits de la réaction (Durán et al., 2002). En plus de ces avantages, les enzymes immobilisées assurent une utilisation en continu puisqu'elle sont lentement libérés du biochar ce qui assure une plus grande efficacité fonctionnelle, une rentabilité, moins de risque de contamination et un meilleur contrôle du procédé.

Plusieurs matériaux de soutien ont été utilisés dans le passé par plusieurs chercheurs pour l'immobilisation d'enzymes. Le support solide le plus largement et couramment utilisé est l'alginate (Datta et al., 2012; Flores-Maltos et al., 2011). L'utilisation de polymères naturels, tels que la chitine et le chitosane est également fréquente (Kapoor and Kuhad, 2007; Vaillant et al., 2000).Les supports d'immobilisation généralement utilisés sont, entre autres, le collagène, la carraghénine, la gélatine, la cellulose, l'amidon, la pectine, la sépharose, les zéolites, la silice et la céramique (Datta et al., 2012). Pour les applications environnementales, telles que le traitement de l'eau, les supports solides pour l'immobilisation d'enzymes doivent être biocompatibles, constituent un microenvironnement de protection et présentent une grande surface spécifique, un gonflement négligeable, une grande stabilité et doivent être rentables. Peu d'études ont rapporté l'utilisation du carbone et du charbon actif comme support solide pour l'immobilisation enzyme (Ganesh Kumar et al., 2010; Ramani et al., 2012). En outre, les lipases et les protéases immobilisées sur du charbon actif ont montré une excellente capacité catalytique, même après 21 cycles de réutilisation pour des applications industrielles alimentaires (Ramani et al., 2012).

Divers procédés d'immobilisation peuvent ainsi être utilisés pour l'immobilisation d'enzymes sur des supports solides. Une figure illustrant ces procédés est donnée dans la figure 1. Pour l'adsorption par le processus d'immobilisation, soit un processus d'adsorption physique ou chimique est utilisé. Le procédé d'adsorption est le résultat des interactions hydrophobes et /ou des liaisons salines entre l'enzyme et le support solide (Datta et al., 2012). L'amyloglucosidase immobilisé sur support de charbon de bois par adsorption présente une activité catalytique de 90% et une excellente efficacité d'immobilisation sur le support solide (Rani et al., 2000). Un autre procédé d'immobilisation largement utilisé est l'association covalente des enzymes sur le support. Des agents de réticulation, tels que le glutaraldéhyde peuvent être utilisé à cette fin. La protection et le maintien des propriétés fonctionnelles et structurales des enzymes sont des défis majeurs pour des agents de réticulation (Datta et al., 2012). Des études antérieures ont rapporté une augmentation de la demi-vie et de la stabilité thermique des enzymes par liaison covalente des supports tels que la silice mésoporeuse et le chitosane (Ispas et al., 2008). Un autre grand procédé d'immobilisation d'enzyme est le piégeage. Dans ce procédé, les enzymes sont mis en cage par des liaisons covalentes ou non covalentes au sein du support solide. Le support solide pour ce type d'immobilisation est généralement des gels ou des fibres (Datta et al., 2012).

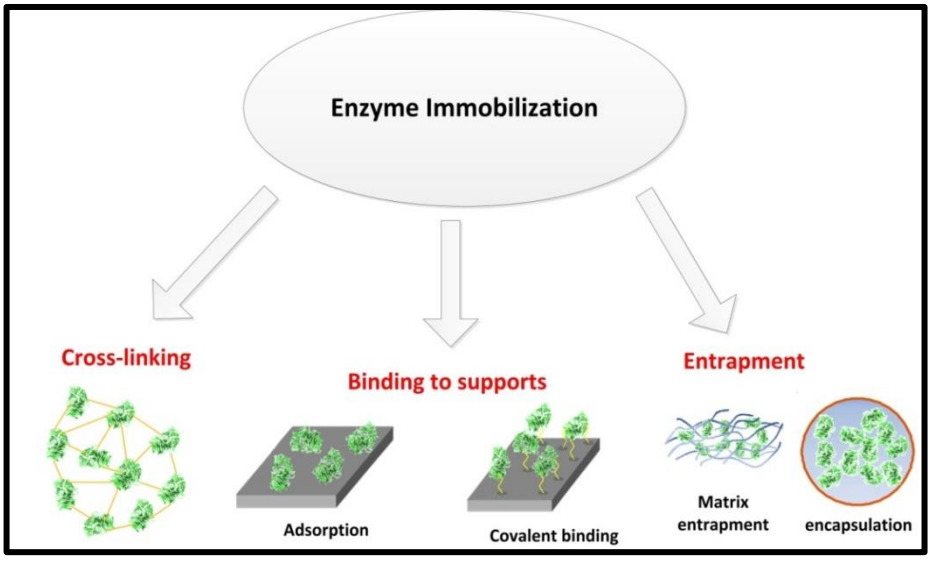


Figure 1: Méthodes d'immobilisation des enzymes

Le microbiochar peut être un excellent support pour l'immobilisation d'enzymes ligninolytiques. Les microparticules de biochar sont biocompatibles, ont une grande surface spécifique, un gonflement négligeable, une grande stabilité et sont très rentables à produire. De plus, le biochar peut adsorber le DCF ce qui va l'éliminer des eaux usées et également va fournir suffisamment de temps pour que les enzymes agissent. En outre, les surfaces copieuses groupes fonctionnels sur le biochar peuvent fournir un support étendu pour des enzymes. L'immobilisation par adsorption et la liaison covalente par des polymères de réticulation peuvent, toutes les deux, être utilisées pour une immobilisation efficace. Par conséquent, l'utilisation de la laccase immobilisée dans des microparticules de biochar peut être une potentielle approche hybride pour l'élimination des produits pharmaceutiques, tels que le DCF dans ce cas.

PARTIE 3: PROBLÉMATIQUE

La problématique de ces travaux de recherche a été identifiée en se basant sur la revue de la littérature :

1. Environ 1443 tonnes de DCF sont consommées dans le monde chaque année et le DCF est considéré comme la 12ème molécule générique meilleur vendu dans le monde entier. En tant que médicament vétérinaire, le DCF peut avoir des effets néfastes sur les consommateurs secondaires, tels que les vautours qui consomment les carcasses de bovins. De plus, les résidus de DCF dans l'environnement, en particulier dans l'environnement aquatique, ont un impact négatif sur plusieurs organismes, tels que les moules et les poissons. Cependant, **les mesures réglementaires / législatives visant à contrôler le DCF dans l'environnement restent minimes.**

2. Indépendamment de l'emplacement géographique, les résidus de DCF ont été détectés dans les eaux de surface ainsi que dans l'eau potable dans le monde entier. De plus, la présence de DCF a également été détectée dans le sol. Dans les stations d'épuration, **la dégradation (dans le système de traitement secondaire) et l'accumulation de DCF dans les boues d'épuration ont**

également été observées à différents niveaux. Le DCF est détectable en très petites concentrations, telles que µg L^{-1} à ng L^{-1} dans les eaux usées. **Les interférences dues aux effets de matrice et aux méthodes analytiques longues sont les principaux défis analytiques de l'analyse du DCF.** Une méthode rapide, sensible et moins encline aux matrices pour le DCF se doit d'être développée.

3. À travers diverses sources, le DCF finit dans une usine de traitement des eaux usées. Étant le principal puits de DCF, l'usine de traitement des eaux usées joue un rôle clé dans l'élimination des DCF de l'environnement. Néanmoins, **dans le système de traitement conventionnel, le DCF n'est pas efficacement éliminé de l'usine de traitement des eaux usées.** En général, environ 30 à 70% d'élimination du DCF sont observés dans les usines de traitement des eaux usées. De plus, les sous-produits potentiels sont rarement étudiés.

4. Au cours des dernières décennies, l'adsorption est apparue comme une méthode de traitement efficace pour l'élimination de divers contaminants organiques émergents, tels que les produits pharmaceutiques et les adsorbants classiques, comme le charbon actif qui a été largement étudié. Cependant, ces études ont été réalisées à des concentrations élevées, telles que le niveau de mg L^{-1}; bien que les contaminants émergents, tels que les DCF sont présents en µg L^{-1} à ng L^{-1}. De plus, les adsorbants sont spécifiques et sélectifs aux contaminants en raison de la porosité et des restrictions de surface. Les nanoadsorbants peuvent être des adsorbants efficaces pour de nombreux contaminants; cependant, le coût de production et les préoccupations récentes sur la toxicité des nanoparticules d'ingénierie sont un inconvénient des nanoadsorbants. En tant qu'alternative à la gestion des déchets, le biocharbon à base de matière carbonée a démontré son potentiel de durabilité et a démontré son utilité en tant que produit à valeur ajoutée pour plusieurs applications environnementales. La réduction de la taille du biocharbon peut être une approche efficace pour l'élimination des contaminants par le processus d'adsorption puisque la réduction de la taille augmentera la surface totale. Avec les microparticules, on peut s'attendre à une augmentation du potentiel d'adsorption par rapport au biocharbon obtenu sous forme brute sans implication de

coût majeur. De plus, la synthèse, la caractérisation et l'utilisation des microparticules de biocharbon pour l'adsorption ne sont pas encore étudiées et des recherches supplémentaires sont nécessaires dans ce domaine. **Les études doivent donc se concentrer sur la recherche d'adsorbants rentables, écologiques et efficaces pour les micropolluants, comme le DCF.**

5. Le traitement des contaminants émergents à l'aide d'enzymes est un nouveau domaine de recherche. Les enzymes ligninolytiques, en particulier la laccase, ont fait preuve d'une excellente capacité de dégradation vis-à-vis des contaminants émergents, tels que les produits pharmaceutiques. Cependant, **pour l'assainissement, de grandes quantités d'enzymes sont nécessaires et par les méthodes conventionnelles, le coût de production peut être très élevé**. Il faut donc étudier d'autres substrats efficaces et rentables pour produire des enzymes.

6. L'utilisation d'enzymes libres pour le traitement des contaminants présente plusieurs inconvénients. **L'utilisation d'enzymes libres n'est pas rentable et les enzymes libres sont non réutilisables et sensibles aux agents de dénaturation**. Par conséquent, l'utilisation de matériel d'appui pour l'immobilisation sera une approche efficace. L'immobilisation des enzymes augmentera sa stabilité et offrira une protection supplémentaire contre la dénaturation par une gamme de co-solvants organiques. En plus de ces avantages que les enzymes immobilisées fourniront, l'utilisation continue de l'enzyme à libération lente et donc une efficacité fonctionnelle accrue et une rentabilité totale est nécessaire. **L'utilisation de microparticules de biocharbon comme support solide pour l'immobilisation enzymatique doit être étudiée**. Les immobilisations d'enzymes sont souvent chargées de défis tels que la résistance au lessivage et à la liaison. Par conséquent, **pour étudier les fixations d'immobilisation (résistance), il faut étudier diverses approches d'immobilisation telles que l'immobilisation adsorptive et covalente.**

7. Les enzymes immobilisées sur le microbiocharbon auront l'avantage supplémentaire de l'adsorption des contaminants par le biocharbon et de la dégradation à médiation enzymatique. De plus, **l'adsorption fournira suffisamment de temps pour l'action des enzymes. Toutefois,**

l'application d'enzymes immobilisées sous forme de lots n'est pas pratique compte tenu des volumes importants d'eaux usées à traiter et de la faisabilité industrielle. Il convient donc d'étudier une alternative appropriée pour le mode d'application des enzymes immobilisées.

PARTIE 4: HYPOTHÈSES, OBJECTIFS ET ORIGINALITÉ

4.1 Hypothèses

«Le développement d'un microsystème imprégné de biocharbon-enzyme (BEMS) pour la dégradation du contaminant émergent le diclofénac » repose sur les hypothèses suivantes :

1. Selon la littérature, la production et la consommation du DCF sont très élevées et les mesures réglementaires/législatives pour contrôler la présence de DCF dans l'environnement sont minimes. **Une méthode rapide, sensible et moins matricielle permet de surmonter les difficultés analytiques liées à la quantification des eaux usées et des boues d'épuration du DCF**. Le DCF n'est pas complètement éliminé dans les stations d'épuration des eaux usées et une grande partie de l'élimination se produit par accumulation dans les boues d'épuration. **L'estimation du DCF à différents stades de la STEP facilitera le suivi du devenir du DCF.**

2. La sélection des matières premières et la méthode de production du biocharbon ont un effet significatif sur les propriétés de ce dernier. **La production et l'application de microparticules de biocharbon peuvent être une méthode rentable, écologique et efficace pour l'élimination des micropolluants.** Les études d'adsorption par des contaminants organiques modèles, comme le bleu de méthylène, peuvent être un outil efficace pour caractériser le potentiel d'adsorption d'un adsorbant. De plus, les échantillons de charbon bio provenant de différentes matières premières et/ou méthodes de production auront une capacité d'adsorption variable vis-à-vis de polluants organiques spécifiques.

3. Le potentiel d'adsorption ou l'efficacité de l'élimination d'un adsorbant dépend de divers facteurs, tels que les propriétés physico-chimiques de l'adsorbant et les conditions environnementales. Par conséquent, tout en étudiant le potentiel d'adsorption du biocharbon sur l'élimination du DCF, **une caractérisation physico-chimique adéquate du biocharbon doit être effectuée.** Les études d'adsorption par lots sont des outils efficaces pour étudier l'adsorption et les interactions adsorbant-adsorbat. **Il est important pour le DCF de réaliser des études d'adsorption à des concentrations pertinentes pour l'environnement.** Plusieurs autres paramètres, comme **le pH et la température, peuvent affecter l'adsorption** et ces paramètres doivent être pris en compte lors des expériences. De plus, les études sur les modèles cinétique et isotherme d'adsorption sont importantes pour comprendre les mécanismes sous-jacents impliqués dans l'adsorption. **Le potentiel d'adsorption peut être amélioré en modifiant la surface du biocharbon et le traitement à l'acide organique peut être un outil efficace pour modifier la surface.**

4. **L'application de matières résiduelles agro-industrielles peut effectivement réduire le coût de production des enzymes. Les enzymes ligninolytiques peuvent dégrader efficacement le DCF, car ce sont des enzymes oxydantes.** Étant donné que les enzymes sont très sensibles aux changements environnementaux, le pH et la température peuvent être pris en compte lors des expériences et ces paramètres doivent être optimisés pour une élimination plus poussée. En outre, il convient d'estimer la quantité d'enzyme nécessaire pour dégrader les concentrations de MDC dans l'environnement. Les produits de transformation de la dégradation doivent être identifiés et la toxicité du DCF et de ses produits de transformation doit être analysée.

5. L'immobilisation des enzymes ligninolytiques sur un support solide améliorera son efficacité et sa stabilité. Les microparticules de biocharbon peuvent être utilisées comme support solide. **Les enzymes ligninolytiques immobilisées sur le biocharbon peuvent être utilisées comme approche thérapeutique efficace pour le DCF. De plus, les études sur l'immobilisation adsorbate et covalente de l'enzyme brute sur les microbiocharbons peuvent illustrer**

davantage les mécanismes de liaison et la force. Cette information peut être utilisée pour étudier la lixiviation et la stabilité des enzymes immobilisées.

6. Les enzymes immobilisées sur les microbiocharbons peuvent également être utilisées pour le développement d'un microsystème imprégné de biocharbon-enzyme. On peut transformer la laccase immobilisée sur microbiocharbon en bioréacteur à lit fixe en comprimant le microbiocharbon avec laccase immobilisé dans une colonne de réaction de dimensions appropriées. **On peut s'attendre à une adsorption et une dégradation continues du DCF à l'intérieur du bioréacteur à lit fixe.** Ce mode d'application peut être facile à utiliser et efficace.

7. Le bioréacteur à colonne de biocharbon avec enzyme immobilisé peut être opéré à grande échelle pour application dans les stations d'épuration des eaux usées. **La production de biocharbon et des enzymes à l'aide de déchets/matières résiduelles peut réduire efficacement le coût.** Par conséquent, dans l'ensemble, le bioréacteur à colonne fixe de biocharbon-enzyme immobilisé présente un potentiel de rentabilité. De plus, l'utilisation de ce système est durable et respectueuse de l'environnement ceci en éliminant les déchets/contaminants aux deux extrémités (production et utilisation). Le bioréacteur à colonne de charbon-enzyme immobilisé peut être opéré à grande échelle de façon efficace et peut être utilisé dans un système de traitement tertiaire dans une station d'épuration des eaux usées.

4.2 Objectifs de recherche

L'objectif global de ce travail est "d'étudier le devenir du DCF dans les STEP et le développement d'un microsystème des enzymes immobilisés sur biocharbon pour le traitement du DCF". Les objectifs spécifiques de l'étude sont décrits ci-dessous :

1. Une méthode rapide de quantification du DCF et du devenir du DCF dans les STEP.

1.1 Une méthode rapide et sensible de détermination du diclofénac dans les eaux usées et les boues d'épuration au moyen de la technique de désorption thermique par diode laser (LDTD) sera mise au point.

1.2 Estimation du DCF dans les échantillons des eaux usées et des boues prélevés à différentes étapes du traitement dans les stations d'épuration de la communauté urbaine de Québec.

1.3 Différentes méthodes d'extraction seront étudiées pour le DCF dans les boues d'épuration.

1.4 Une étude comparative d'une méthode existante de chromatographie liquide (CL) avec la méthode LDTD nouvellement mise au point pour les échantillons environnementaux à matrice élevée sera effectuée.

2. Préparation de microparticules de biocharbon, sa caractérisation pour le potentiel d'adsorption par le composé organique modèle, le bleu de méthylène.

2.1 Les microparticules de biocharbon seront préparées à partir de biocharbon en utilisant différentes méthodes de production.

2.2 Le potentiel d'adsorption de microparticules de biocharbon sera étudié en utilisant un contaminant organique modèle, le bleu de méthylène.

3. Efficacité d'adsorption des microparticules de biochar pour l'enlèvement de DCF

3.1 L'efficacité de l'élimination du DCF des microparticules de biocharbon préparées à partir de diverses matières premières (bois de pin et lisier de porc) sera étudiée.

3.2 La cinétique, la thermodynamique et les effets de l'adsorption sur le pH seront étudiés.

3.3 Des modèles isothermes d'adsorption seront étudiés pour comprendre les mécanismes sous-jacents de l'adsorption et la caractérisation physico-chimique des microparticules de biocharbon.

4. Fonctionnalisation verte du microbiocharbon pour l'élimination améliorée du DCF

4.1 Différents acides organiques (acide acétique, acide citrique, acide tartrique, acide oxalique et acide malique) à différentes concentrations et en utilisant la méthode catalytique par agitation ainsi qu'en autoclave seront testés pour la fonctionnalisation efficace des microbiocharbon (biocharbons de bois de pin et de porc et biocharbons de coquille d'amande).

4.2 Des microbiocharbons fonctionnalisés seront testés pour l'élimination du DCF.

5. Production de l'enzyme laccase et son utilisation pour la dégradation du DCF

5.1 Les résidus agro-industriels seront utilisés comme substrats pour la production de la laccase.

5.2 Le profilage de production sera effectué et divers inducteurs seront testés pour une production améliorée de laccase.

5.3 Des études de dégradation du DCF seront effectuées dans diverses conditions expérimentales en utilisant l'enzyme brute produite (enzymes libres non purifiées sans aucune immobilisation).

5.4 Les produits de transformation du DCF par dégradation enzymatique seront identifiés.

6. Immobilisation adsorptive de la laccase brute concentrée sur microbiocharbons

6.1 L'immobilisation adbsorbante de la laccase brute sera étudiée dans le cadre de diverses activités de la laccase afin de tracer les isothermes d'adsorption et de découvrir ainsi les mécanismes sous-jacents.

6.2 La cinétique de désorption de laccase sera étudiée pour étudier les mécanismes de désorption ainsi que la force de liaison adsorbant.

6.3 La laccase immobilisée sera utilisée pour la dégradation du DCF.

7. Laccase brute d'immobilisation covalente sur microbiocharbon

7.1 Les microbiocharbon de bois de pin, de fumier de porc et de coquille d'amande ainsi que leurs dérivés fonctionnalisés de l'acide citrique seront utilisés pour l'immobilisation covalente de la laccase avec le glutaraldéhyde comme agent de réticulation.

7.2 Les microbiocharbon immobilisés par la laccase seront soumis à des tests pour l'élimination de la DCF.

7.3 Les stabilités du pH, de la température, du stockage et du fonctionnement seront étudiées pour les enzymes immobilisées.

8. Le bioréacteur à colonne à lit compact avec laccase immobilisé sur biocharbon pour le traitement de la DCF

8.1 Des essais d'efficacité d'adsorption du réacteur à colonne à lit rempli de biocharbon seront effectués au moyen d'études d'adsorption.

8.2 Le bioréacteur à colonne de lit rempli de microbiocharbon avec laccase immobilisée sera testé pour l'élimination continue du DCF.

8.3 Une évaluation préliminaire de l'estimation des coûts et de la faisabilité industrielle pour le bioréacteur à colonne à lit compact avec laccase immobilisé sur biocharbon.

4.3 Originalité du travail

En se basant sur des hypothèses et des objectifs, l'originalité de la présente étude est la suivante :

1. Mise au point d'une méthode analytique novatrice, rapide et moins dépendante de la matrice pour le DCF (LDTD-MS/MS) qui réduira le temps d'analyse de quelques minutes à quelques secondes.

2. Préparation de nouvelles microparticules de biocharbon qui étaient aussi efficaces que le charbon actif à des concentrations plus faibles de contaminant organique modèle sans aucune activation/modification de surface.

3. L'efficacité des microparticules de biocharbon de bois, de pin, de lisier et de porc sera étudiée pour l'élimination du DCF dans diverses conditions expérimentales, et les mécanismes d'adsorption impliqués seront étudiés. Les études pour l'élimination du DCF par adsorption sur biocharbon sont minimes. De plus, le fumier de porc et les microparticules de biocharbon en bois de pin peuvent être des adsorbants efficaces pour le DCF en raison de leurs propriétés de surface spécifiques, ce qui n'a jamais été étudié auparavant. Par conséquent, les mécanismes d'adsorption sont inconnus et doivent être étudiés.

4. Une nouvelle méthode de fonctionnalisation verte pour les microbiocharbons utilisant des acides organiques.

5. Stratégie de production rentable pour produire de la laccase en utilisant des résidus agro-industriels comme substrats et amélioration de la production de laccase à l'aide de divers inducteurs. Les résidus agro-industriels de résidus de marc de pomme, de pulpe, de déchets solides de papier et de fibres d'alfa n'ont jamais été étudiés auparavant en tant que substrats pour la production de laccases utilisant les champignons, *Trametes versicolor*. Dégradation du DCF induite par la laccase dans diverses conditions expérimentales et identification des produits de transformation a été effectuée. L'utilisation de la transformation DCF à médiation laccase n'a jamais été étudiée auparavant. De plus, les connaissances sur la cinétique, les effets du pH et de la température et les produits de transformation de la transformation DCF à médiation laccase sont minimes.

6. L'immobilisation des laccases brutes sur divers microbiocharbons (bois de pin, fumier de porc et coquille d'amande) n'a jamais été étudiée auparavant. Le mécanisme sous-jacent d'adsorption ainsi que la désorption de laccase sur toute surface de biocharbon est encore inconnu. La présente étude

examine ces mécanismes ainsi qu'une application potentielle de laccase immobilisée, soit l'enlèvement du DCF.

7. L'immobilisation covalente peut éventuellement améliorer la stabilité de la laccase immobilisée puisque les fixations chimiques sont le principal mécanisme en cause. De plus, la fonctionnalisation de l'acide citrique peut augmenter la liaison covalente entre laccase et biocharbon. Ces deux aspects n'ont jamais été étudiés auparavant et cela implique la nouveauté.

8. Le concept d'un bioréacteur à colonne à lit compact avec laccase immobilisée sur biocharbon, écologique et durable pour le traitement de la DCF dans les eaux usées est nouveau. De plus, ce bioréacteur à colonne peut être implanté dans l'étape tertiaire de la station d'épuration des eaux usées.

*Dans l'ensemble, l'originalité de la thèse est la suivante, "**L'enlèvement d'un médicament prioritaire - diclofénac des eaux usées à l'aide de nouveaux microsystèmes imprégnés de biocharbon-enzyme".***

PARTIE 5: SOMMAIRE DES DIFFÉRENTS VOLETS DE RECHERCHE EFFECTUÉS DANS CETTE ÉTUDE

Cette section a été divisée en quatre sous-sections. La première section présente les résultats en ce qui concerne le devenir de DCF dans une usine de traitement classique des eaux usées. De plus, cette section présente également les résultats d'une méthode rapide et novatrice qui a été développée pour la quantification des DCF dans les boues et des eaux usées. La deuxième section illustre les propriétés d'adsorption de divers microbiochars. Particulièrement, la suppression du DCF a été étudiée par adsorption sur divers microbiochars et acides organiques microbiochars fonctionnalisés ont été testés pour l'élimination améliorée du DCF. La troisième section examine la production rentable de l'enzyme lignolytique «laccase» pour la dégradation du DCF. Finalement, la

technique d'adsorption, ainsi que des procédés d'immobilisation covalente de laccase sur les microbiochars ont été étudiés dans cette section.

5.1 Le devenir de DCF dans les stations d'épuration et la méthode novatrice et rapide pour la quantification du DCF dans des eaux usées et des boues d'épuration

5.1.1 Méthode rapide et sensible pour la quantification du DCF dans les eaux usées et les boues d'eaux usées par désorption thermique de la diode laser - couplée à la spectrométrie de masse en tandem (LDTD- MS / MS)

La quantification des micropolluants dans la matrice complexe de l'environnement est souvent difficile analytiquement. Une méthode fiable, rapide et sensible sur la base de l'ionisation chimique et de désorption thermique de la diode laser / pression atmosphérique (LDTD / APCI) couplée avec spectrométrie de masse tandem (MS / MS) a été développée pour la quantification du DCF dans les boues d'eaux usées et des eaux usées. La technique LDTD-APCI-MS / MS nouvellement mise au point réduit le temps d'analyse de 12 secondes au lieu de 12 minutes pour la méthode classique LC-ESI-MS / MS. Les limites de détection de la méthode (dans de l'eau MilliQ) pour la méthode LDTD-APCI-MS / MS ont été calculé 270 ngL^{-1}(LOD) et 1000 ngL^{-1} (LOQ). La limite de détection de la méthode (MDL) a été chiffrée à 300 ngL^{-1} pour des échantillons d'eaux usées et 75 ng g^{-1} pour les échantillons de boues d'eaux usées. En outre, deux procédés d'extraction ont été comparés, l'extraction assistée par ultrasons (USE) et l'extraction accélérée par solvant (ASE) pour traiter le DCF à partir de boues des eaux usées et la méthode ASE s'est avéré plus efficace avec un taux de récupération de 95 ± 7% comparativement au procédé USE avec 84 ± 5% de récupération. L'approche LDTD remplace la séparation chromatographique par désorption thermique de la diode laser, et permet donc d'éviter l'utilisation d'un solvant organique. Ceci constitue un saut vers des principes de la chimie verte et réduit la matrice d'analyse relativement à l'approche de LC.

5.1.2 Le destin et la répartition des DCF à la Communauté urbaine épuration des eaux usées au Québec

La nouvelle méthode a été utilisée pour la quantification ainsi que l'étude du devenir du DCF dans l'usine de traitement des eaux usées des collectivités urbaines du Québec. Un pourcentage d'enlèvement d'environ 76% a été obtenu avec le système de traitement conventionnel de la station d'épuration des eaux usées. Lors de l'analyse de la répartition du DCF dans WWS et WW, DCF présentait une affinité envers WW comparativement aux WWS. La concentration en matières solides a été observée de 10 ± 0,5 gL^{-1} et de 8 ± 0,3 gL^{-1} pour la boue primaire et la boue secondaire, respectivement. Par conséquent, l'élimination ou la dégradation aux niveaux primaire et secondaire a été calculée. Environ 18% d'élimination / dégradation a été observée à l'étape de dessablage. Environ 20% de cette concentration ont été accumulées dans la phase de dégradation de boues et 13% ont été observées. Le reste de la DCF a été transférée au système de traitement secondaire. Le système secondaire a montré une dégradation ultérieure de 31% de la concentration reçue et le surplus a été accumulé dans les boues et les eaux usées. Par conséquent, en dehors de l'élimination ultérieure des boues, une dégradation globale d'environ 50% a été observée dans la station d'épuration.

En conclusion, l'enlèvement par traitement du DCF n'a pas été très efficace et des traces de DCF restant peuvent être potentiellement toxiques pour plusieurs organismes. Consécutivement, des études ultérieures ont été planifiées pour un meilleur traitement des DCF pour une élimination complète.

5.2. Identification et caractérisation des microbiochars et des études d'adsorption pour l'élimination du DCF

5.2.1 Préparation et caractérisation des microbiochars

Le Biochar peut être considéré comme un excellent adsorbant pour divers contaminants, y compris les micropolluants en raison de ses caractéristiques de surface. Des microparticules de biochar peuvent être préparées en utilisant un procédé économiquement viable. Le bleu de méthylène a été utilisé comme modèle d'adsorption pour la caractérisation du potentiel d'adsorption préparée microbiochar. Dans cette étude, les microparticules préparées à partir de trois différents types de biochar présentaient des capacités d'adsorption améliorées (0,975 mg g^{-1}) avec des concentrations réduites de 10 mgL^{-1}, ce qui était comparable avec le charbon actif disponible dans le commerce (0,980 mg g^{-1}) utilisé pour l'étude. Les capacités d'adsorption de biochar varient avec le matériau source et le procédé de préparation. La capacité d'adsorption du bleu de méthylène pour le colorant cationique a également augmenté avec l'augmentation de la surface des microparticules. La capacité d'adsorption maximale (Q_{max}) a été estimée à 3,99 mg g^{-1}, 16,30 mg g^{-1}, et 1,66 mg g^{-1}, respectivement pour le bois de pin, le fumier de porc et de papier provenant des microparticules de biochar à une concentration MB de 50 mg L^{-1}. Les modèles isothermes d'adsorption Langmuir et Freundlich ont été utilisés pour indiquer l'adsorption du bleu de méthylène. Les données d'équilibre ont été bien décrites par le modèle Langmuir pour le modèle BC-PM et BC-PD et Freundlich pour BC-PW. À des concentrations plus faibles, les microbiochars ont montré d'excellents rendements d'élimination pour le bleu de méthylène qui a servi comme un excellent adsorbant pour les micropolluants. Les modèles isothermes d'adsorption Langmuir et Freundlich ont été utilisés pour signaler l'adsorption du bleu de méthylène. Les données d'équilibre ont été bien décrites par le modèle Langmuir pour le modèle BC-PM et BC-PD et Freundlich pour BC-PW.

5.2.2 Adsorption de diclofenac sur différents microbiochars

Les microbiochars ont été préparés à partir de matières premières de bois de pin (BC-PW) et de fumier de porc (BC-PM). L'adsorption du DCF sur BC-PM a été jugée efficace sur BC-PW. À la suite des études antérieures sur la caractérisation des microbiochars, ces derniers peuvent être des excellents adsorbants de polluants organiques à des concentrations plus faibles. À propos de l'efficacité de l'élimination, un taux de 99,6% a été observé pour BC-PM à une concentration de l'environnement correspondant à 500 µg L^{-1}. Pour BC-PW, l'adsorption a été expliquée par le modèle pseudo de premier ordre alors que pour BC-PM par modèle pseudo-second ordre. Pour les deux adsorbants, la diffusion intrapparticulaire a été jugée le principal mécanisme expliquant le comportement d'adsorption. L'adsorption sur BC-PW a été en grande partie affectée par le pH, alors que la dépendance du pH d'adsorption de BC-PM était minime. Le comportement thermodynamique des deux adsorbants nous mène à suggérer que l'adsorption de DCF sur BC-PW était endothermique alors qu'elle est exothermique pour BC-PM.

5.2.3 Méthode écoresponsable (verte) pour la fonctionnalisation du biochar et l'adsorption du diclofénac

Après avoir démontré que la microbiochars peut être utilisée comme un excellent adsorbant pour les micropolluants et le DCF en particulier, nous avons testé le biochar activé. Malgré des résultats d'élimination très satisfaisants, ce procédé implique l'usage de composés chimiques toxiques. Les biochar produisent à partir de biomasse, tel que le bois de pin, de fumier de porc, et la coquille d'amande ont été fonctionnalisés avec de l'acide acétique (CH_3COOH), l'acide citrique ($C_6H_8O_7$), l'acide tartrique ($C_4H_6O_6$), l'acide oxalique ($C_2H_2O_4$) et l'acide malique ($C_4H_6O_5$). Deux méthodes de fonctionnalisation, une simple agitation pendant la nuit à température ambiante et l'autoclavage ont été testées et comparées pour l'efficacité de fonctionnalisation. Pour le biochar en bois de pin, on a trouvé que l'acide citrique est plus efficace de l'acide acétique (augmentation d'environ 40% des groupes -COOH sur la surface). Les acides tartrique et oxalique se sont révélés efficaces pour le

fumier de porc biochar. Cependant, les effets des deux étaient minimes sur fonctionnalisation. Les acides citrique et oxalique se sont montrés efficaces pour la coquille d'amande biochar et l'acide citrique relativement plus efficace (augmentation d'environ 45% des groupes -COOH sur la surface). La composition de Biochar (matière première) avait un effet significatif sur l'efficacité de fonctionnalisation alors que pour la réaction avec les acides organiques, les surfaces de biochar ont répondu de manière différente avec tous les acides. En outre, la concentration (molarité) d'acides a eu peu ou pas d'effets sur l'ampleur de la fonctionnalisation. Une simple agitation sur une longue période a été jugée efficace comparativement à l'autoclavage à une température élevée pour courte période. Dans l'ensemble, on constate que l'acide citrique est adéquat pour une fonctionnalisation efficace (COOH) sur la surface de biochar. La fonctionnalisation d'acide citrique a augmenté au total des groupes fonctionnels acides de 23,6%, 10,2% et 26,2% pour le bois de pin, fumier de porc et la coquille d'amande, respectivement. Les biochars fonctionnalisé avec l'acide citrique à donner une élimination significative ($p < 0,05$) du DCF à l'équilibre.

5.3 La production et l'immobilisation de laccase sur les microbiochars pour le retrait du diclofenac

5.3.1 Production de laccase par voie agro-industrielle et de la dégradation de diclofénac

Les enzymes lignolytiques et la laccase, en particulier, ont montré leur potentiel pour la restauration de plusieurs contaminants à haute concentration. Cependant, les méthodes classiques de production sont généralement coûteuses. Dans cette étude, les résidus agro-industriels ont été utilisés comme substrats pour la production de laccase. Les résidus de l'industrie de jus de pomme, purée de pommes (49,16 ± 2,5 U/gds) et de déchets solides de l'industrie de pâte et papier (52,4 ± 2,2 U / gds) se sont révélés les substrats les plus efficaces pour la production de laccase par rapport aux fibres sèches (alfa14,26 ± 0,8 U / gds). Le sulfate de cuivre a été jugé efficace comme inducteur de production de laccase sur tween 80, l'alcool vératrylique le rouge de phénol. Cependant, l'effet inducteur des éléments étudiés a été plutôt limité avec tous les supports. La laccase brute produite à

partir d'agro-éléments a été utilisée pour la dégradation du DCF. Pour des concentrations dans l'environnement (500 µg L^{-1} de DCF), pH de 4,5 et à une température de 50°C, on a retrouvé a été, la dégradation de DCF avec laccase est jugée efficace. Pour la dégradation médiée par la laccase, la vitesse de réaction a été jugée d'ordre zéro à des concentrations de substrat supérieur et de premier ordre, à des concentrations de substrat inférieures (pertinentes pour l'environnement). Dans des conditions favorables, la dégradation a été observée jusqu'à 99% pour DCF dans les 5 heures. Les produits de transformation initiaux ont été identifiés par LC-MS / MS et identifiés comme 3'-hydroxydiclofenac, 4'-hydroxydiclofenac, et 5-hydroxydiclofenac. Le mécanisme de dégradation de DCF catalysée par laccase a été proposé comme un déclenchement de cycle d'hydroxylation et de minéralisation finale de CO_2, NH_3, H_2O et du chlore.

5.3.2 Immobilisation par adsorption de laccase brute sur les microbiochars

Même si la laccase d'origine agro-industrielle a montré une excellente efficacité pour la dégradation des DCF, l'application de laccase sous forme libre fait face à des défis en ce qui concerne l'application pratique en raison de problèmes de stabilité liés à l'enzyme. L'immobilisation d'enzymes sur des supports solides peut être une excellente mesure corrective. L'immobilisation de laccase brute adsorbate sur le bois de pin (BC-PW), le fumier de porc (BC-PM) et la coquille d'amande (BC-AS) microbiochars a été étudiée en utilisant les principes de la chimie physique et à travers les isothermes. Avec tous les biochars, comme l'activité initiale de laccase de l'augmente dans la solution brute, la capacité de liaison a également augmenté et par conséquent l'efficacité d'immobilisation. BC-PM a été jugée la plus efficace (31,4 ± 3,1 U g^{-1}) à 10 U mL^{-1} d'activité enzymatique suivie par BC-AS (24,3 ± 4,8 U g^{-1}) et BC-PW (14,58 ± 3,3 U g^{-1}). L'adsorption monocouche homogène conçoit le mécanisme clé de la liaison enzyme-biochar. La laccase immobilisée possède une viabilité et une stabilité 3 fois plus élevée que celle de l'enzyme libre en termes de durée d'exposition. La laccase immobilisée est capable de dégrader le diclofenac avec près de 100% d'efficacité au bout de 5 heures en considérant des concentrations semblables à celles retrouvées dans l'environnement (500 µg L^{-1}).

5.3.3 Immobilisation covalente de la laccase brute sur les microbiochars

Un second mode opératoire d'immobilisation; l'immobilisation covalente a également été étudiée pour l'immobilisation efficace et améliorée de la laccase. En partie (concentré) la laccase brute purifiée a été immobilisée de manière covalente sur BC-PW, BC-PM, et BC-AS en utilisant 5% de glutaraldéhyde. BC-PM (34,1 ± 1,1 U g^{-1}) a montré la capacité de liaison la plus élevée de la laccase suivie BC-AS (25,3 ± 2,8 U g^{-1}) et BC-PW (16,18 ± 0,3 U g^{-1}). Le prétraitement à l'acide citrique a amélioré la capacité de liaison de la laccase avec tous les microbiochars et a montré une amélioration de 20% de la liaison Laccase et BC-AS. La surface améliorée et du CEC biochars à base biochar de fumier de porc comparativement au biochar de bois a généré une meilleure liaison de laccase sur BC-PM. Cependant, le biochars dérivé du bois (BC-PW et BC-AS) ont montré une meilleure stabilité de pH, température, de stockage et de stabilité opérationnelle relativement aux biochars dérivés de fumier (BC-PM). Le complexe laccase microbiochars immobilisées a été utilisé pour l'élimination des micropolluants, en particulier le diclofénac et l'élimination complète de cet élément a été observée au bout de 2 heures sous le mode discontinu avec 0,5 g de BC-PM lié laccase à une concentration pertinente de l'environnement de 500 µgL^{-1} dans les effluents des eaux usées.

5.4 Élimination du Diclofenac par le bioréacteur sur colonne à lit fixe

L'application des enzymes immobilisées sous forme de lot est pratiquement non viable en tenant compte des grands volumes d'eaux usées qui doivent être traitées. Le bioréacteur sur colonne à lit fixe peut être une alternative. L'adsorption continue et la dégradation des DCF peuvent être considérées à l'intérieur du bioréacteur à lit fixe. Ce mode d'application peut être ergonomique et efficace. Les microbiochar préparés à partir de bois de pin (BC-PW) fumier de porc (BC-PM) et la coquille d'amande (BC-AS) ont été évalués pour l'élimination des DCF à des concentrations pertinentes en environnement, dans des conditions de fonctionnement continu de colonne à bobines fixes. BC-PM s'est avéré un adsorbant efficace sur BC-PW et BC-AS et qui a été attribué à la zone

de surface plus élevée, la porosité, et la présence de divers groupes fonctionnels contenant des métaux. À une concentration dans l'environnement (500μg L^{-1}), BC-PM présentait une capacité d'adsorption de 4,10 mg g^{-1} de masse de lit: 2 g, débit: 2 mL min^{-1} et à pH 6,5. Pour tous les biochars, l'adsorption du DCF dans une colonne à lit fixe était dépendante de la masse d'adsorbant (hauteur du lit) et de la concentration du DCF. La capacité d'adsorption diminue de plus en plus avec la hauteur du lit tandis qu'elle augmente avec la concentration de DCF. Les modèles Thomas et Yoon-Nelson ont été appliqués avec succès pour prédire les courbes de percée, ce qui indique que ces modèles ont été utiles pour la conception et la mise à l'échelle de biochar en colonnes à lit fixe.

Le biochar lié à la laccase a démontré une efficacité de traitement ou d'enlèvement plus élevé sur le biochar non lié et une amélioration significative dans le temps de pénétration a été observée contribuant ainsi à la biodégradation du DCF dans la colonne. Le microsystème (BEMS) imprégné biochar-enzyme a montré un potentiel de rentabilité et de valorisation des déchets à partir de déchets ou de matières résiduelles pour le développement de BEMS.

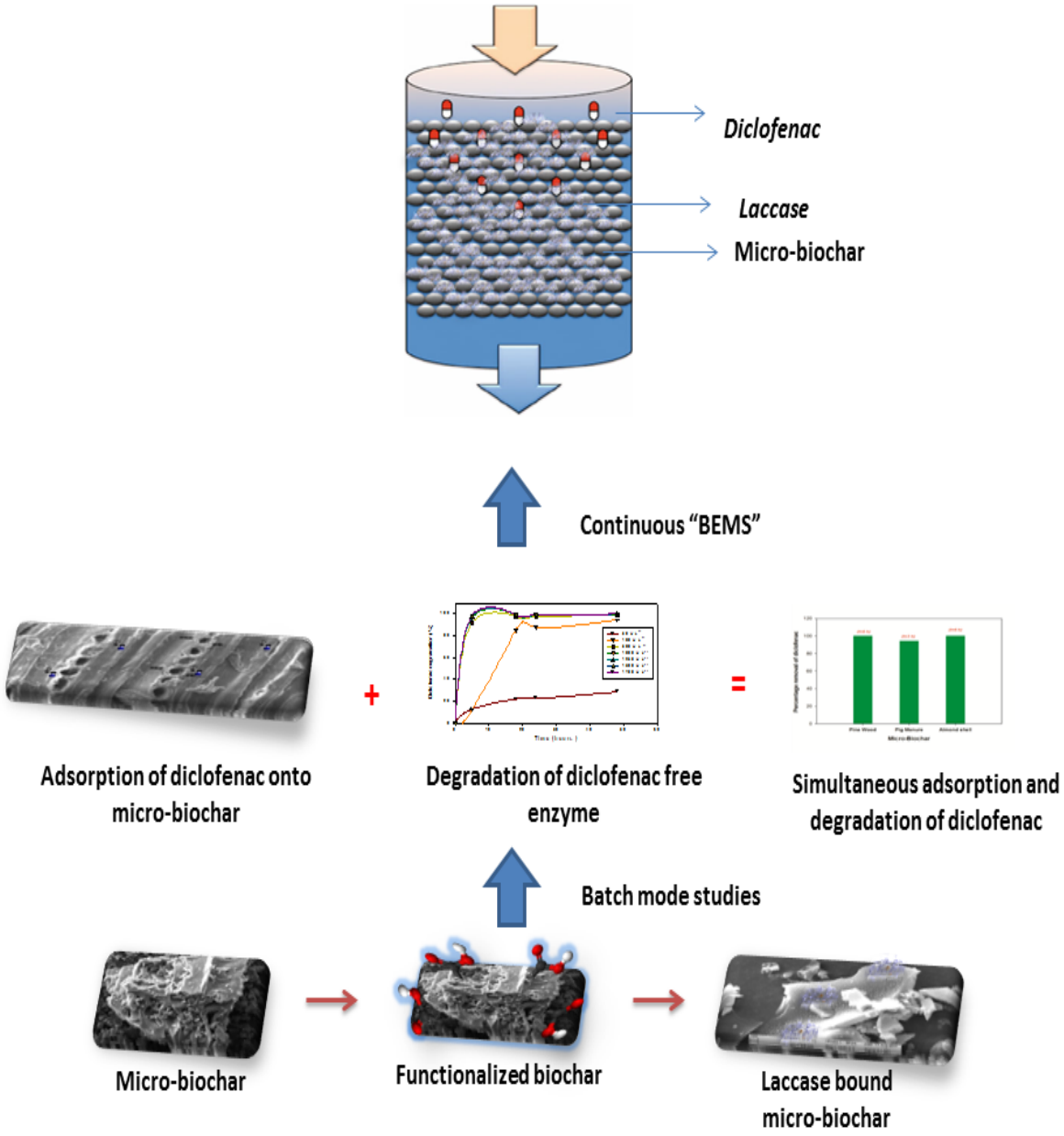


Figure 2: Principales étapes dans le travail de recherche

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CHAPTER 2

DICLOFENAC: OCCURRENCE, FATE AND ANALYSIS

PART 1

DICLOFENAC AND ITS TRANSFORMATION PRODUCTS: ENVIRONMENTAL OCCURRENCE AND TOXICITY - A REVIEW

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RÉSUMÉ

Le Diclofenac (DCF) est un anti-inflammatoire commun utilisé à travers le monde. Les recherches intensives menées au cours des dernières décennies ont confirmé la présence ubiquitaire du DCF dans différents milieux environnementaux. Sa présence fréquente dans les cours d'eau fraîches et sa toxicité potentielle envers différents organismes tels que les poissons et les mollusques font du DCF un contaminant environnemental émergent. Aux concentrations généralement mesurées dans l'environnement, le DCF ne présente pas d'effet toxique pour les organismes vivants, cependant l'exposition chronique peut mener à des effets plus sévères. Pour le DCF, la principale méthode d'élimination sont les systèmes d'épuration conventionnels des eaux usées qui sont en mesure de réduire la concentration de 30 à 70%. La fraction non traité se retrouve par la suite dans les eaux de surface. Le DCF peut interagir avec différent contaminants dans l'environnement et dans les stations d'épuration des eaux. Ces contaminants sont les métaux, la matière organiques et même les métabolites associés au DCF. Ces interactions peuvent mener à la possible création de d'autres contaminants émergents. Dans le contexte actuel, le destin environnemental du DCF dans les différents milieux tel que le sol et l'eau a été adressé avec un survol des méthodes de traitement actuelles. De plus, les préoccupations liées à la toxicité du DCF dans les milieux aquatiques et terrestres ainsi que les voies de dégradation abiotiques sont aussi discutées. Des études supplémentaires sont requises pour mieux évaluer le destin et les effets écotoxicoligiques du DCF et ses métabolites. Ces études devront considérer les interactions possibles du DCF avec d'autres contaminants afin de développer une méthode de traitement efficace pour le DCF jusqu'à des concentrations traces.

Mots clés : Diclofenac; destin environnemental; toxicité; interactions; métabolites; contaminants émergents

ABSTRACT

Diclofenac (DCF) is a prevalent anti-inflammatory drug used throughout the world. Intensive researches carried out in the past few decades have confirmed the global ubiquity of DCF in various environmental compartments. Its frequent occurrence in freshwater environments and its potential toxicity towards several organisms such as fish and mussels makes DCF an emerging environmental contaminant. At typical detected environmental concentrations, the drug does not exhibit toxic effects towards living organisms, albeit chronic exposure may lead to severe effects. For DCF, about 30-70% removal has been obtained through the conventional treatment system in wastewater treatment plant being the major primary sink. Thus, the untreated DCF will pass to surface water. DCF can interact with other inorganic contaminants in the environment particularly in wastewater treatment plant, such as metals, organic contaminants and even with DCF metabolites. This process may lead to the creation of another possible emerging contaminant. In the present context, environmental fate of DCF in different compartments such as soil and water has been addressed with an overview of current treatment methods. In addition, the toxicity concerns regarding DCF in aquatic as well as terrestrial environment along with an introduction to the metabolites of DCF through consumption as well as abiotic degradation routes are also discussed. Further studies are required to better assess the fate and toxicological effects of DCF and its metabolites and must consider the possible interaction of DCF with other contaminants to develop an effective treatment method for DCF and its traces.

Keywords: Diclofenac; Environmental fate; Toxicity; Interactions; Metabolites; Emerging contaminant

1. Introduction

Pharmaceutical industry has emerged as one of the largest and prominent industry worldwide. Large amount of pharmaceuticals of different categories are being used to cure and care human and animal health. In general, pharmaceuticals comprise compounds which include materials extensively used in medicine, agriculture and biotechnology, such as drugs, antibiotics and hormones. The worldwide average per capita consumption of pharmaceuticals per year is estimated to be about 15 g. In industrialized countries, the usage is even as high as 50 to 150 g (Alder et al. 2006). Pharmaceutically active compounds (PhACs) are one of the conspicuous classes of pharmaceuticals which by one route or another, enter the environment as the parent compound or as pharmacologically active metabolites (Halling-Sørensen et al. 1998). It is estimated that worldwide consumption of active compounds amounts to 100,000 tons or more per annum (Kummerer 2004). Usually, drugs are developed with an intention of having a beneficial biological effect on the organism to which they are administered, though many such compounds will often pass into the environment where they may exert an unwanted biological effect (Halling-Sørensen et al. 1998). The global occurrences of pharmaceuticals and PhACs in aquatic environment have been arising as a problem with unknown consequences. PhACs have been reported to be present in different environmental compartments and often the short term as well as long term effects are obscure (Kunkel and Radke 2012; Langford et al. 2011) Hence, it has been relatively recently that PhACs have become a subject of interest to environmentalists worldwide (Hao et al. 2007).

Among PhACs, non-steroidal anti-inflammatory drugs (NSAIDs) are widely used throughout the world and detected in different environmental compartments at concentrations ranging from ng L^{-1} to low mg L^{-1} (Halling-Sørensen et al. 1998; Khetan and Collins 2007). Moreover, NSAIDs are over-the-counter (OTC) drugs in most of the countries and this in turn increases the chances for consumption and hence, their presence in the environment. DCF is often recognized as the ‘world’s most popular pain killer’ and is also the most commonly used NSAID, with a market share close to that of the next three most popular drugs combined (ibuprofen, mefenamic acid, naproxen) (McGettigan and Henry

2013). The name diclofenac is derived from its chemical name: 2-(2,6-dichloranilino)phenylacetic acid. Diclofenac was discovered by Ciba-Geigy, a Swiss pharmaceutical company in 1973 (now merged to Novartis). Diclofenac is commonly used to reduce inflammation and to relieve pain in diseased conditions, such as arthritis or acute injury. It also works as antiuricosurics and analgesic. DCF can be applied to skin or it can be administered orally. DCF is supplied as or contained in medications under a variety of trade names. In Canada, DCF is sold as Voltaren Emulgel and some other common names are Votalin (China), Diclofenaco Normon (Spain), Volini (India), Diclofenac-Asteria (USA and Korea), Diclo-Denk (Germany) Voltaren (Argentina, Australia, Belgium, Egypt, France, Germany, Israel, New Zealand, Norway, Portugal, Russia, South Africa, Sweden, Turkey) (sources - www.drugs.com/diclofenac, www.drugbank.ca, www.scbt.com).

Pharmacological and physico-chemical properties of DCF are listed in Table 1. Often DCF is not completely removed from wastewater treatment plants (WWTP) due to its poor degradation and higher consumption rates (Fatta-Kassinos et al. 2011; Zorita et al. 2009). Hence, DCF is frequently detected in rivers, sediments and sludges (Kunkel and Radke 2012; Langford et al. 2011). Relatively recently, DCF has drawn much more attention due to its frequent occurrence in drinking water sources (Gros et al. 2010) and its potential harmful effects on many organisms at significant concentration (Cleuvers 2004; Oaks et al. 2004).

Diclofenac is normally used as salt of sodium or potassium for improved solubility and absorption. Until date, no literature is exclusively available on the environmental perspectives and concerns regarding the drug, DCF. Most of the previously published reviews have discussed the fate of diclofenac in WWTPs (Vieno and Sillanpää 2014; Zhang et al. 2008). The objective of this chapter is to briefly summarize the current status of diclofenac in the environment, review the available information about its consumption, occurrence, toxicity, resistance, persistence and metabolites. In addition this review addresses the hypothetical possibility of potential interactions of DCF with other organic and inorganic contaminants, emerging contaminants along with its own metabolites. Major

research gap in the current knowledge and future research need in diclofenac fate and transport in environment have also been highlighted.

2. Global consumption

It is fairly impossible to calculate the exact global consumption of diclofenac because of various reasons, such as use of different trade names for DCF, use for human and veterinary purposes and that the drug is an over the counter drug. Nevertheless, Zhang et al. (2008) estimated that about 940 tons of diclofenac is consumed globally on an annual basis from Intercontinental Marketing Services (IMS) health data (Zhang et al. 2008). About 877 tons of diclofenac were sold in 2007 in 76 major countries which are believed to account for about 96% of the global diclofenac pharmaceutical market (Zhang et al., 2008). In a 2012 report from “Fierce Pharma”, diclofenac was listed as the 12th bestselling generic molecule globally. The total sales of diclofenac in 2011 were estimated to be $1.61 billion dollars with a sales charge of 15.5% annually (Palmer 2012). While considering the sales change information along with previously carried-out consumption estimation, there is high probability that the annual consumption of DCF might have crossed 1000 tons. Moreover, recent reports suggests that along with conventional and developed markets, such as United States, emerging markets such as India, China and Brazil also consume more than 60 tons of DCF on annual basis (Acuña et al. 2015). In addition, the consumption estimation of DCF does not cover the veterinary consumption due to non-availability of data and hence the consumption can be even higher while considering the veterinary usages.

Diclofenac is included in the emergency medical list (EML) of 74 countries. The exact annual consumption of diclofenac in North America is not available, even though in North America, the market shares of DCF in the drug market are on a continuous rise. In US, DCF contributes to about 5-6% of the total NSAID market while in Canada, 17% of NSAID consumed is DCF (Henry 2013). According to the current trend, consumption of DCF will keep on increasing in North America since

lifestyle diseases, such as arthritis and heart diseases are now becoming common and the also the ageing population will require medicines, such as pain killers.

The annual consumption or prescription data for diclofenac is available for some countries. According to consumption estimation models, in Australia, it was estimated that 4 tons of DCF was used annually (Khan and Ongerth 2004). In Europe, the largest user of DCF is Germany with 86 tons of usage in the year 2001 (Huschek et al. 2004). For rest of the European countries; the consumption comprises for England 26.13 tons per year (Jones et al. 2002) , Austria 6.14 tons per year (Strenn et al. 2004) and France 16 tons per year (Ferrari et al. 2003). The total consumption of DCF in the entire European continent was estimated to be 179.8 tons per year (Ferrari et al. 2003). For most of the Asian and African countries, data on consumption of DCF is not available due to the lack of studies on consumption and also due to the absence of inventory of sales. With the frequent reports on toxicological effects observed in these countries on vultures, it can be assumed that the consumption can be colossal.

Recent studies based on IMS health data (which serves 82% of the global population) from 86 countries estimated that at present on an average 1443 ± 58 tons of DCF is consumed globally (Acuña et al. 2015). In this study authors also indicated that 39.5% of DCF was consumed in Asia and 28.7% Europe. However, this is only an indication on the consumption DCF for human health related applications and does not include the consumption of DCF for veterinary uses. At present it is impossible to calculate the total consumption of DCF since the data for veterinary consumption is not available.

3. Legislation

DCF in environment has been lately acknowledged to constitute a health risk to terrestrial organisms. DCF is extremely toxic to vultures (even though they do not consume DCF directly) and its use on cattle has wiped out and threatened vulture populations in India, Pakistan and Nepal since the vultures consume cattle carcasses. India was the first country to bring in regulations on the

consumption of DCF. In 2006, the manufacture and veterinary use of DCF was banned in India (The Drug controller general 2006). Further in 2008, India placed additional restrictions on diclofenac for animal use, with contravention punishable with imprisonment. Further, the same year, Nepal and Pakistan banned the drug for veterinary use followed by Bangladesh in 2010 (K.Venkateshwarlu 2011).

For the veterinary use, diclofenac does not have a central marketing approval from the European Medicines Agency (EMA) and it is authorized independently in each member state. Further, EMA has set a Maximum Residue Limit for DCF in bovine and porcine species. Since 2013, the commercial production of DCF started in Spain and Italy and is being exported to other European Union countries. A coalition of famous organizations including the Vulture Conservation Foundation, The Royal Society for the Protection of Birds, Bird Life Europe and the IUCN Vulture Specialist Group are campaigning for an EU-imposed, continent-wide ban on veterinary diclofenac following the lessons from India (BirdLife 2013; Tavares 2014a; Tavares 2014b). Diclofenac has been recently added to Environmental Quality Standards (EQS) of Europe. According to the European Community document (COM(2011)876), the annual average value of EQS (evaluation of quality standards) for DCF was 0.1 μg L^{-1}. However, this document has been amended and DCF is put on watch list until next review (Johnson et al. 2013). In 2013, DCF has been selected for inclusion in the watch list of "EU Water Framework Directive" in order to collect sufficient monitoring data for the determination of risk reduction measures. According to this proposed EQS document , the maximimum alloable concentrations are 0.1 μg L^{-1} in fresh waters and 0.01 μg L^{-1} in marine waters . Another European coutry, United Kingdom (UK) has placed DCF in 'the list of priority substances' which forced the water industries to search technologies to remove DCF from wastewater.

Regulatory measures for the use of DCF has been imposed by few countries. Apart from very few regional/country wise regulations in global scale scenario, strict legislation or directives does not exist to regulate the production and consumption of DCF to control environmental presence of diclofenac. Since DCF is emerging as a contaminant of concern and has been acknowledged to

pose health risks towards terrestrial organisms, such as vultures, recent efforts have focussed on survey on contamination of diclofenac in the environment and drafting the rules for the regulation of the drug. However, there is a need to control the consumption of drug and thereby reduce the environmental presence. Besides, it is important to set appropriate directives, such as setting a maximum concentration limit in the environment.

4. Environmental fate

Even though the general purpose of the pharmaceuticals is to positively affect human or animal health in a unique manner, they often have some adverse effects on the environment. When these pharmaceuticals enter the environment, they may affect the same pathways in animals having identical or similar target organs, tissues, cells or biomolecules (Fent et al. 2006). Studies have demonstrated the potential adverse effects of diclofenac in the environment (Cleuvers 2004; Gros et al. 2010; Kunkel and Radke 2012; Oaks et al. 2004). Hence, it is important to understand the origin and fate of pharmaceuticals and diclofenac in particular in the environment for designing appropriate pollution remedial measures.

Figure 1 demonstrates the possible entry routes of DCF to the environment. Being an anthropogenic pollutant, the source of diclofenac is the drug industry, and it is used for both human and veterinary purposes. Through both human and veterinary routes, DCF ends up in wastewater treatment plants or in landfills as DCF or its metabolites. Also, there are fewer but considerable probabilities for the drug to reach wastewater treatment plants directly from pharmaceutical industrial residues. Likewise, the conventional treatment process of DCF in wastewater treatment plants are ineffective (Fatta-Kassinos et al. 2011; Zorita et al. 2009) and hence DCF can end up in the surface water and the possibility for the percolation of DCF to the drinking water sources cannot be overruled. In addition, percolation probabilities for DCF from landfills to surface water are fairly higher. At present, none of the studies reported DCF causing major risks to aquatic life at an environmentally relevant concentration. The potential detrimental effect of DCF in the aquatic environment has been revealed

from many studies (Cleuvers 2003; Fent et al. 2006; Jones et al. 2002; Lee et al. 2011). The no effect concentration (NEC) of DCF was calculated to be 100 µg L^{-1} for freshwater cladocerans and Japanese medaka (Lee et al. 2011). Another study on rainbow trout and zebra fish , two typical examples for freshwater organisms no observed effect concentration (NOEC) was observed to be 320 µg L^{-1} (Memmert et al. 2013).

4.1 Removal processes

The conventional treatment system including wastewater treatment plants exhibited a moderate to higher degradation efficiency of DCF. DCF is moderately persistent in the environment. In the reviewed studies, the maximum removal obtained was about 93% by adsorption on activated carbon followed by ozonation (Beltran et al. 2009). Primary treatment was also efficient with coagulating and flocculating agents, such as $FeCl_3$, $Al_2(SO_4)_3$ but did not remove the drug completely from wastewater (Carballa et al. 2005)**.** Conventional activated sludge processes showed better efficiency when compared to the MBR. On an average, 30-70% removal can be obtained by the existing removal methods (Vieno and Sillanpää 2014; Zhang et al. 2008). An overview of the treatment methods is given in Table 2. The removal efficiency mostly depends upon the treatment methods. For instance, the conventional activated sludge process exhibited about 75% removal (Kimura et al. 2005; Lonappan et al. 2016), however mostly this removal was achieved through sorption to the sludge. This happened in conventional wastewater treatment plant where a certain percent of DCF removal occurred through the accumulation of the drug in wastewater sludge (Lonappan et al. 2016). Hence, the effective removal or degradation of the drug is minimal in conventional wastewater treatment system. Several other treatment options which are based on sorption process have been suggested by various researchers recently (Sotelo et al. 2014; Sotelo et al. 2012; Suriyanon et al. 2013). Even though various natural and synthetic adsorbent materials, such as activated carbon, biochar, silica based polymer based adsorbents exhibited excellent removal efficiency towards DCF, as a whole, these processes cannot be considered as sustainable methods; as they do not completely remove DCF from the environment. Instead, water/wastewater bound DCF is

accumulated on the adsorbent. Another most widely used method for the treatment of DCF is based on advanced oxidation processes, such as ozonation (Beltran et al. 2009). Few recent developments are promising and sustainable. Studies demonstrated the effective use of enzymes for the complete degradation of DCF from water (Marco-Urrea et al. 2010) which will create no harmful byproducts.

4.2 Presence in aquatic environment

The potential detrimental effect of DCF in the aquatic environment has been revealed from many studies (Cleuvers 2003; Fent et al. 2006; Jones et al. 2002; Lee et al. 2011). However, all these studies were conducted at laboratory scale. In surface water bodies, DCF has been detected in ng L^{-1}whereas in wastewater; the concentration has been as high as micrograms per liter. The concentration decreased by natural processes, such as soil retention, biodegradation and photo-transformation and also by physico-chemical processes in wastewater treatment plants. Table 3 presents the recent occurrence of diclofenac in the aquatic environment in different countries over a time period of 15 years. In surface waters, DCF contamination occurred in rivers, estuaries and lakes (Buser et al. 1998; Kim et al. 2007; Metcalfe et al. 2003; Öllers et al. 2001). Also, there are a few reports of detection in groundwater and drinking water (Benotti et al. 2008; Rabiet et al. 2006). Most of the detection has been from EU countries, but this does not indicate that DCF was only present in European countries. In Asia, the available data was insufficient to predict the environmental concentrations as there was no systematic annual data. When compared to Europe, the consumption of DCF in North America was lower. However, there are a few reported cases of DCF detection in the environment (Metcalfe et al. 2003; Sosiak and Hebben 2005).

In reviewed data, the highest concentration detected was in rivers and was in Pakistan as 4900 ng L^{-1} (Scheurell et al. 2009) and one of the potential reason could be due to the absence of advanced WWTPs in Asian countries. Meanwhile, recent reports from Canada showed that WWTP effluents contain very high concentration of DCF such as 16 µg L^{-1} (Lonappan et al. 2016). German water bodies have also been heavily polluted with DCF and maximum concentration detected was 1030

ng L^{-1} in river water (Heberer 2002a). In Spain, DCF residues were detected in river Delta (Lopez-Serna et al. 2013). DCF residues were detected in almost all European Union countries (Hernando et al. 2006; Loos et al. 2010). In US and Germany, DCF was even detected in drinking water which warrants attention (Benotti et al. 2008; Heberer 2002b).

DCF has been detected mostly in fresh water bodies all over the world. Water containing DCF is discharged to surface water from WWTPs after treatment. The concentration ranges from few hundreds of ng L^{-1} to thousands of ng L^{-1}. In Asian countries, there are no strict measures to monitor the concentration of DCF in aquatic environment. From the few available studies in rivers, it is clear that DCF is discharged to surface water without proper treatment. Therefore, in this region, the load of DCF into the aquatic environment could be reduced by proper treatment of wastewater. Mostly, these wastewater treatment plants must be equipped with train of primary, secondary and tertiary treatment for an effective removal.

4.3 Presence in soil

There are a few reported cases of detection in DCF in soil. Diclofenac could potentially reach agricultural lands through the application of municipal sewage sludge as a source of nutrients in soil or through wastewater and it has been detected in the Canadian province of Ontario (Al-Rajab et al. 2010). Studies on the sorption coefficient of diclofenac proved that sorption even in sandy sediment was relevant and therefore diclofenac was less mobile in groundwater (Scheytt et al. 2005). On the other hand, studies from Israel reported that DCF showed slower mobility in organic rich agricultural soils and higher mobility in fresh water column and which caused its leaching to the ground water and ultimately to drinking water after rain events (Chefetz et al. 2008; Drillia et al. 2005). Few other studies also pointed to the same possibility. (Chefetz et al. 2008; Xu et al. 2009)

The toxicity of DCF in soil towards plants and soil organisms/microorganisms has been poorly understood. More investigations must be carried out in this environmental compartment. The only information that is available is that DCF has been readily degraded in soil and was highly adsorbed

by organic rich soil (Al-Rajab et al. 2010; Xu et al. 2009). DCF is less toxic towards leguminous plants when compared to other pharmaceuticals, such as sulfamethazine (Ziółkowska et al. 2014) and DCF does not show any harmful effect towards plant growth (Carter et al. 2014b). Also, DCF does not show any toxic effects (behavior, weight change, mortality) towards soil organisms, such as earthworms (Carter et al. 2014a). However, recent reports on soil application of wastewater sludge containing DCF suggested medium risks towards soil microbes (Verlicchi and Zambello 2015).

From the little available information on the fate and toxicity of DCF in soil, DCF exhibits lower toxicity and moderate persistence. However, in soils with large amount of organic matter, DCF gets adsorbed to the soil and exhibited resistivity towards aerobic/anaerobic degradation and may leach out to the groundwater causing accumulated toxic effects. Thus, future studies should focus on the fate of DCF in agricultural lands with further monitoring of fate through groundwater aquifers.

5. Toxicity

Earlier, most of the studies on the toxic effects of DCF were focused on its adverse effects on the aquatic animals. The toxic concerns regarding DCF in freshwater environment have been studied in the laboratories with the help of model organism for toxicological studies. The first widely noted case of pharmaceutical causing major ecological damage was the sudden collapse of vultures due to the consumption of carcasses containing residues of DCF. After these consecutive incidents in the first decade of 20^{th} century, DCF has got much worldwide attention. The following section describes the toxic concerns over DCF in aquatic environment; particularly in freshwater environment and the events of DCF toxicity towards terrestrial animals.

5.1 Aquatic organisms

Many toxicity studies were conducted worldwide to evaluate the toxicity of DCF in aquatic organisms. One of the widely used and highly standardized methods for measuring toxicity was the acute immobilization tests. (Ferrari et al. 2003), conducted one of the first studies on the toxic effects of DCF. This study was carried out on bacteria, algae, microcrustaceans and fish, and showed relatively less toxic effects even at environmental concentrations. On the contrary, later studies revealed the potential impacts of diclofenac on the environment. According to risk assessment studies, the potential ecological risk of diclofenac in surface waters was higher (Hernando et al. 2006). (Cleuvers 2004), conducted ecotoxicity studies using acute *Daphnia* and algal tests and revealed that DCF was potentially harmful to aquatic organisms. In the same study, Cleuvers (2004) also reflected that under field or environmental concentrations, the adverse effects were either less or negligible and a mixture of pharmaceuticals can be considerably toxic even at lower concentrations. In crustacea (*Daphnia magna* sp.), at acute concentrations, such as mg L^{-1}, DCF induced high mortality rate. From different studies for 48h exposure, the presence of DCF produced higher mortality and the EC_{50} values were reported to be 22.4 mg $L^{-1}$and 39.9 mg L^{-1}(Ferrari et al. 2003; Haap et al. 2008). Also, for *Ceriodaphnia dubia* sp., mortality was observed and the EC_{50} was 22.7 mg L^{-1} (Ferrari et al. 2003). On the contrary, (Lee et al. 2011) reported 3 times higher values for 48 h EC_{50} tests for the same species. Surprisingly, studies from Canada reported that DCF was a major risk even at predicted environmental concentrations (10-100 ng L^{-1}) (Lawrence et al. 2007). These studies in river biofilm communities revealed the significant impacts of DCF on community structure and function at even lower concentrations of 100 ng L^{-1}.

DCF was known to exert deadly effects by damaging renal and gastrointestinal tissue in several vertebrates, such as fishes. In an exposure assessment study, (Letzel et al. 2009) found that at environmentally relevant concentrations, such as nanograms per liter, DCF may cause chronic adverse effects on fish populations. In fish, Japanese medaka (*Oryzias latipes)*, DCF adversely affected the growth in egg phase and resulted in significant reduction of hatchability and delay in

hatching (Lee et al. 2011). In a study on zebra fish, (Hallare et al. 2004) observed the same results. Hatching was delayed when the embryos were exposed to 2000 µg L^{-1}. In brown trout, DCF was not completely excreted through first pass metabolism, but a significant part of the DCF entered enterohepatic circulation. The resulting prolonged availability of DCF in the organism possibly promoted accumulation of DCF (Hoeger et al. 2008). For the same species, a heavy damage of gill, liver and kidney was observed at 50 µg L^{-1} (Hoeger et al. 2005). For rainbow trout, even at environmentally observed concentrations, DCF interfered with the biochemical functions and lead to tissue damage (Mehinto et al. 2010; Schwaiger et al. 2004). DCF may accumulate in liver kidney, gills and muscle tissues of rainbow trout and can cause cytological alterations even at 1 µg L^{-1} (Schwaiger et al. 2004; Triebskorn et al. 2004).

Mussels got affected by DCF at concentrations that are prevalent in the environment. At nanograms per liter level concentrations, DCF significantly induced lipid peroxidation (LPO) in mussels indicating tissue damage (Schmidt et al. 2011). A relatively recent study (Gonzalez-Rey and Bebianno 2014) proved that at 250 ng L^{-1}, which came very close to the concentrations in certain German rivers, DCF induced tissue specific biomarker responses leading to the tissue damage. DCF was also adversely affecting the metabolism and growth of the blue mussels which are common in Baltic Sea (Ericson et al. 2010).

Except for mussels, environmentally relevant concentrations appeared to be less toxic for aquatic animals. Most of the studies suggested that continuous exposure to DCF even at very low concentrations may lead to some adverse effects in aquatic animals. It was estimated that the no effect concentration of DCF was 0.1 mg L^{-1}(Lee et al. 2011), which is very high when compared to those observed in aquatic systems/ real environmental conditions (Table 3). Moreover, DCF and its metabolites were observed in fish bile (Kallio et al. 2010). Several photo-transformation products can be more toxic than DCF at the concentration that may come close to the environmental concentrations (Schmitt-Jansen et al. 2007). However, the toxicity of metabolites of DCF was poorly understood in the environment. The future studies may include the toxicity studies of photo-

transformation products of DCF and mixture toxicity studies. Also, the future studies should concentrate on the bioaccumulation of DCF in the food web and chronic exposure studies at lower but environmentally relevant concentrations since the DCF residue is continuously introduced and dynamically increasing in the environment.

5.2 Terrestrial organisms

The first widely noted case of pharmaceutical causing major ecological damage was the sudden collapse of vultures due to the consumption of carcasses containing residues of DCF, and this threatened several vulture species to extinction (Oaks et al. 2004; Taggart et al. 2007b). DCF was the major cause of collapse of population of three Gyps vulture species (*Gyps bengalensis, Gyps indicus, Gyps tenuirostris*) which were severely affected, reduced by 98% in the Indian sub-continent and was included in "critically endangered" species list of IUCN (Das et al. 2010).

In 2003, studies reported the catastrophic collapse of Indian white-backed *Gyps bengalensis* and long-billed *Gyps indicus* vulture populations due to some unknown reason or epidemics (Ferrari et al. 2004; Prakash et al. 2003). However, the exact reason for this sudden collapse was discovered by Oaks et al in 2004, and it was found to be the renal portal vasoconstriction caused by DCF. On the contrary, another study reported the cause of death as decreased uric acid excretion (Naidoo and Swan 2009). Most of the investigations led to the probability of renal failure due to the consumption of DCF (Sharma et al. 2014; Swan et al. 2006; Taggart et al. 2007a). The major food source of vultures was the livestock from cows and goats. These animals were treated with DCF and although DCF was short lived in these animals, the prevalence in carcasses available to vultures may still be very high (Taggart et al. 2007a) and Gypes vultures were extremely susceptible to very lower doses of DCF (Swan et al. 2006). These events even affected the ecosystem. DCF did not only affect the population of vultures but also the community structure of the ecosystem. Vultures are keystone species and their decline has a range of socio-economic as well as culture and biodiversity impacts. As an example, the rabies causing dogs and vultures were having same food source. The

decrease in the number of vultures increased the availability of food for dogs by reducing competition over food (Markandya et al. 2008). Hence, the decline in vultures had a biological and social effect in the specific region of concern.

The veterinary application of DCF is also threatening the vulture species in Africa (Naidoo et al. 2009; Virani et al. 2011). There are reported cases of major decline in the abundance of vultures and other scavenging raptors from Kenya and the reason was suspected to be DCF (Virani et al. 2011). Moreover, relatively recently, DCF has been reported to be fatal for eagles which widens the diversity of raptors threatened by DCF (Sharma et al. 2014).

Recent studies reported that the ban of DCF for veterinary use in south Asian countries was an effective measure and the vulture population is on rise now (Khadka and Mandal 2013; Prakash et al. 2012). Hence, legislative measures succeeded for DCF in South Asia through its ban for veterinary use. Nevertheless, there are DCF residues already present in the environment, especially those that pass through the soil into groundwater which need to be investigated to avoid any future chronic toxicity effects on organisms. However, there are other terrestrial animals which feed on the carcasses of cattle and which need to be studied as they are the intermediate transporters of DCF during its veterinary use.

6. Metabolites

In animals, after consumption, DCF is mostly degraded into hydroxyl derivatives. DCF is easily degraded to its transformation products in the environment. The major natural process of degradation is photo-transformation by sunlight. DCF is one of the best investigated pharmaceutical residues in the environment (Vieno and Sillanpää 2014; Zhang et al. 2008). However, studies on the occurrence and toxicity of its metabolites in the environment are not well understood.

6.1 Via consumption routes

In human body, DCF derivative is found in urine and plasma. The hydroxylated and methoxylated derivatives of DCF are present in their free forms well as glucuronide-conjugated forms. In an earlier study, Stierlin et al. identified the metabolites of DCF in human body and the main metabolite was identified to be 4'-hydroxydiclofenac (2-[2,6-dichloro-4 hydroxyphenylamino] phenylethanoic acid) (30%) and other major metabolites are 5'-hydroxydiclofenac (2-[2,6-dichlorophenylamino]-5-hydroxyphe nylethanoic acid) (10 %) , 3'-hydroxydiclofenac (2-[2,6-dichloro-3-hydro xyphenylamino] phenylethanoic acid) and 4',5-dihydroxydiclofenac (2-[2,6-dichloro-4-hydroxyphenylamino]-5-hydroxyphenylethanoic acid) (15%) (Boettcher et al. 1991; Stierlin et al. 1979)**.** Recently, some minor metabolites were also identified in human body (Stulten et al. 2008a). Along with hydroxyl derivatives of DCF, acyl glucuronide and hydroxyl acyl glucuronide were also found in mouse and fish (Kallio et al. 2010; Naisbitt et al. 2007).The identified human metabolites of DCF are depicted in table 4. The wide occurrence of human metabolites of diclofenac in water and its structural similarity towards diclofenac is a matter of concern on toxicity grounds and needs to be investigated further.

6.2 Via abiotic degradation route

DCF was readily degraded in the sunlight. The half-life of DCF is estimated to be 3.3 h (Schmitt-Jansen et al. 2007). DCF followed first order kinetics during photodegradation and was detected in water cycle. (Qin and Yang 2012). Photodegradation was identified as the main removal process for the degradation of DCF in lakes and it was estimated that about 90% of DCF was eliminated by this process (Buser et al. 1998). Many phototransformation products of DCF were identified by various researchers (Agüera et al. 2005; Moore et al. 1990; Qin and Yang 2012). Most significant process of DCF phototransformation was identified as the photocyclisation to the corresponding monohalogenated carbazole (Eriksson et al. 2010). Most of the photochemical decomposition products occurred as two main sub-structures: 2-Chloro- and 2,6-Dichlorodiphenylamine derivatives and also 8-Hydroxy- and 8-Chlorocarbazole derivatives (Agüera et al. 2005; Moore et al. 1990).

Not only diclofenac, but also its metabolites are globally entering the aqueous environment. There are reports that some of the DCF metabolites are even more toxic than DCF. Some of the phototransformation products exhibited a six fold increase in toxicity in algal reproduction tests (Schmitt-Jansen et al. 2007; Schulze et al. 2010). In view of the toxic effects of diclofenac on several water organisms, it appeared that the metabolites also initiated objectionable reactions in other organisms and needed strict surveillance during drug toxicological and environmental monitoring experiments (Stulten et al. 2008b). Therefore, future studies need to investigate the potential toxicity of DCF metabolites. However, the analysis of DCF transformation products is often laden with challenges. For example, unavailability of metabolite standards obstructs the exact quantification of DCF transformation products. Another analytical challenge is the low level (often nanograms per liter) occurrence of the drug and its transformation products. Hence, MS/MS instruments with high sensitivity and appropriate methods are needed to quantify this "micro-pollutant".

7. Interactions with other pollutants- proposed approach

For diclofenac, WWTPs being one of the major sink; interaction with other contaminants is a possibility that has to be studied. A hypothetical representation of a typical secondary treatment system in wastewater treatment plant depicting interaction of diclofenac with other pollutants is shown in Figure 2. Municipal wastewater treatment plants are complex systems that receive contaminants from a variety of sources. These include contaminants, such as suspended solids, biodegradable organics such as proteins, carbohydrates and fats (particularly in hospital wastewater), nutrients, such as nitrogen, phosphorus and carbon -(from various origin), refractory organics, such as pesticides, phenols, surfactants, heavy metals, dissolved organics, and pathogens of various category (bacteria, viruses, protozoa etc.). In addition, they also receive various pharmaceuticals and personal care products (PPCPs) termed as emerging contaminants. Often emerging contaminants are detected in minute concentrations, such as ng L^{-1} or µg L^{-1}. These lower concentrations are even undetectable without specific sensitive methods. Moreover, for

pharmaceuticals, corresponding metabolites are formed during *in vivo* human/veterinary metabolism and they could become a new category of potential emerging contaminants.

Micro-pollutants, such as pharmaceuticals are present in 'micro' concentrations. However, even at this micro concentration, they can cause adverse impacts in the environment. In addition, these contaminants can interact/combine/aggregate with other pollutants of the same or different class. For example, for pharmaceuticals, including DCF, it is proven that in a mixture of pharmaceuticals, considerable combination effects could also occur if some or even all substances were applied in concentrations below their NOEC (No Observed Effect Concentration) (Cleuvers 2004; Cleuvers 2008).

As described in section 5, many studies already evaluated the toxic concerns regarding DCF in various environmental compartments. However, very few studies have examined the mixture toxicity concerns regarding DCF along with other pharmaceuticals. A recent study suggested antagonistic interactions between nutrients and emerging contaminants including DCF in stream biofilms (Aristi et al. 2016); however long term real exposure experiments are required to bring out actual mixture toxicity effects. Hence, to interpose realism into future risk assessment studies, mixture toxicity studies must focus on: (i) whether ecotoxicity of a pharmaceutical mixture is higher than that of the toxicity of each individual pharmaceutical; and (ii) if acting singly at lower concentrations what will be the toxicity of the individual drug, DCF (Backhaus 2014). These studies will thus need efforts from environmental exposure assessment studies for toxicity evaluation. Furthermore, since the number of drugs and its varieties are on an increase along with hundreds of existing pharmaceuticals, the need for creating database for the mixture toxicity concerns of DCF along with other pharmaceuticals is vital. These interactions and mixture toxicity studies must consider toxicokinetic and toxicodynamic interactions in test organisms along with ecological interactions for real scale studies (Backhaus 2014) .

7.1 Proposed interactions of DCF with metals, other inorganics and organics

Conventionally, diclofenac treatment occurs in the wastewater treatment plant. WWTP receive wastewater from various sources, such as industries, hospitals, households and municipal wastewater, among others. Because of the differences in origin, the contaminants are also diverse. Metals and heavy metals, in particular is a major class of contaminants that are usually present (Barakat 2011; da Silva Oliveira et al. 2007; Karvelas et al. 2003). Various physico-chemical conditions in a conventional wastewater treatment plant can catalyze this process. Previous studies already proved that DCF along with certain metals possess higher anti-inflammatory activity and antioxidant property than DCF alone (Kovala-Demertzi 2000). Moreover, DCF has active groups such as amino, hydroxyl, carbonyl, and carboxyl groups in its structure. These groups can enhance the metal complexation/binding properties with most of the metals. Hence, organometallic complexation of drugs via chelation is a possibility that can happen in WWTP. DCF can act as ligand coordinated to the metal ions via the deprotonated functional groups present on it. Moreover, DCF complexes with Hg(II), Pb(II), and Sn(II) are already known to be antibacterial agents (Refat et al. 2014) while Cu(II) complex of DCF can cleave DNA (Theodorou et al. 1999). Hence, on reaction with metal complexes, the properties of DCF can change completely and become another potential pollutant possessing antibacterial and cell destruction capacity. Until date, most of the metal complexation studies of DCF commonly have been used to uncover its novel therapeutic values and underlying mechanisms. However, to the best of our knowledge, none of the studies focused on DCF metal complexes and its toxicity concerns in wastewater effluent. Hence, while considering DCF as an emerging contaminant having potential toxic concerns towards several organisms, the metal complexes of DCF must be considered as an emerging contaminant and must be treated with care while considering its antibacterial properties. The metal complexes of DCF add another chemical complexity raising toxicity concerns for several organisms.

Moreover, because of the structural properties, DCF can act as a ligand for other inorganic elements/groups. This property of DCF, particularly in WWTP is crucial from toxicological point of

view. Unfortunately, none of the published studies investigated these aspects on DCF toxicity. Theoretically, the interactions with other inorganic pollutants, such as sulfates, nitrates chlorides are a certain possibility for DCF along with possible complexation/ aggregation with other numerous organic pollutants in wastewater matrix

7.2 Proposed interactions of DCF with other ECs and DCF metabolites

Although mixture toxicity concerns with other pharmaceuticals are already established and studied, the toxicity concerns over DCF metabolites are still valid. The major metabolites of DCF are hydroxy-metabolites (3'-hydroxydiclofenac, 4'-hydroxydiclofenac, 5'-hydroxydiclofenac). The mixture toxicity effects of these metabolites are still unknown. There is a possibility for the mixture toxic effect of DCF and these metabolites similar to one reported with other pharmaceuticals. In addition, various conditions existing in WWTP can catalyze the effective combination of these metabolites and DCF, and it may leads to the creation of another potential contaminant. In addition, about 65% percent of DCF is present as DCF metabolites (Boettcher et al. 1991) and this increases the availability for all metabolites and DCF.

The reported metabolites of DCF have -OH groups at various positions in the DCF structure. Hence, the basic molecular structure of DCF largely remains unaltered. The presence of hydroxyl groups on the structure can enhance the interactions with metals through π- π interactions. (Yamada et al. 1990); applied this principle to DCF metabolites which can easily form metal complexes since the DCF metabolites are the hydroxyl derivatives of DCF. Moreover, due to the presence of active groups, such as amino, hydroxyl, carbonyl, and carboxyl groups in metabolites of DCF, there is a possibility of interaction between these molecules. Hence, these possibilities point toward creation of "new emerging contaminant" of unknown properties. Likewise, for DCF, it is possible to have interactions with other ECs. Several other ECs, such as pesticides, surfactants, PPCPs are present in wastewater (Petrović et al. 2003). Often these ECs are compounds having several active groups in their structure. Thus, it is possible to have multiple interactions with one or many other ECs and

DCF along with its metabolites. These interactions could be with other ECs and/or its metabolites/transformation products during treatment process." Recently, the synergistic effect of DCF and its nitrogen transformation products were studied along with sulfamethoxazole and its transformation products (Osorio et al. 2016) suggesting that contribution of these compounds to overall toxicity of complex environmental samples, should not be dismissed.

Hence, experimental evidence is necessary for these interactions to perform toxicity studies and drafting adequate risk assessment for DCF. However, the existing ecotoxicological and environmental exposure data is not sufficient and realistic while considering WWTP as the major sink for DCF. Future studies, particularly toxicity focus on these mixtures of ECs, metabolites, metals and DCF that are either proven or likely to form new products. To further increase the realism of ecotoxicological studies, investigation based on these approaches is necessary with overwhelming number of emerging contaminants being added each day.

8. Conclusions

Diclofenac is one of the major PhACs which has a far flung usage throughout the world. The residues of diclofenac are found worldwide in surface, ground and drinking water. Even though diclofenac is removed by natural processes, such as photodegradation, the residue still remains in the environment as potential toxic metabolites and as diclofenac. Diclofenac in the environment is detected in lower concentrations, such as nanograms per liter to micro grams per liter and from the available ecotoxicology data, it is apparent that these lower concentrations can cause acute toxic effects to many organisms, such as mussels. At lower measured concentrations, there are fewer chances of acute toxicity. However, extended exposure to lower concentrations may lead to chronic toxicological effects In the case of diclofenac, continuous entry into the environment due to the year –round use of medication is increasing the diclofenac residue in the environment. The fate of diclofenac in soil is poorly understood. In soils, with large amount of organic matter, diclofenac gets adsorbed to the soil and shows resistivity towards aerobic/anaerobic degradation and may leach out

to the groundwater causing toxic effects. The toxicity of diclofenac metabolites is not well investigated and some studies suspected that few metabolites can be potentially more toxic than the parent compound. There still remains a lacuna for investigating the environmental impact of metabolites and also the toxicological effects if any, on the flora and fauna. Diclofenac can interact with other inorganic contaminants, such as metals, organic contaminants and even with diclofenac metabolites as they all are present in a complex wastewater matrix in wastewater treatment plant. This process may lead to the formation of another possible emerging contaminant. Further studies are required to better assess the fate and toxicological effects of diclofenac and its metabolites and must consider the possible interaction of diclofenac with other contaminants to develop an effective treatment method for diclofenac and transformation products. Moreover along with DCF, DCF metabolites must be considered as another emerging contaminant and treatment methods must emphasize metabolites as well. The tertiary treatment system must be equipped with more advanced treatment methods, such as advanced oxidation and environmental friendly enzymatic treatment methods which are known to be effective for several contaminants including DCF.

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Table 1: Physico-chemical and pharmacological properties of diclofenac (in unionized form)

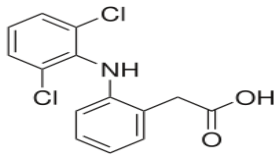
Properties		Reference
Structure		www. pubchem.ncbi.nlm.nih.gov, www.chemspider.com
Molecular formula and molecular weight	$C_{14}H_{11}Cl_2NO_2$, 296.16 g mol^{-1}	www.drugbank.ca, www.pubchem.ncbi.nlm.nih.gov
CAS no.	15307-86-5 15307-79-6 (disodium salt)	www.drugbank.ca, www.pubchem.ncbi.nlm.nih.gov
Water solubility	2.37 mg L^{-1} (25 °C)	www.drugbank.ca, www.chemspider.com
Henry's law constant	4.79×10^{-7} Pa m^3 mol^{-1} (25 °C)	www.scbt.com, www.pubchem.ncbi.nlm.nih.gov
Melting and boiling points	283-285 ^{0}C and 412 °C at 760 mm Hg (Predicted) respectively	www.drugbank.ca, www.chemspider.com
p*K*a	4.15	www.drugbank.ca, www.chemspider.com
Log K_{ow} (logarithm of octanol-water partition coefficient)	4.51	www.scbt.com, www.pubchem.ncbi.nlm.nih.gov

Table 2: Removal of diclofenac in wastewater treatment plants

Major Treatment method	Process conditions and other treatment methods	Removal %	Comments	Reference
Submerged MBR	Membrane flux 0.4 m^3/ml/d HRT 9h MLSS conc- 10,000 mg L^{-1}	40 %	--------	(Kimura et al. 2005)
Conventional activated sludge	HRT 13h MLSS conc- 1700 mg L^{-1}	75%	--------	(Kimura et al. 2005)
Adsorption on activated carbon followed by ozonation	--------	> 93%	activated carbon reduced toxicity	(Beltran et al. 2009)
Conventional biological wastewater treatment	--------	65%	average result of study on 5 biological wastewater treatment plants in Spain	(Gomez et al. 2007)

Primary treatment (coagulation and flotation)	Coagulation- $FeCl_3$,$Al_2(SO_4)_3$ PAX Flotation- 12 ^oC - 25^oC	40-70% 20-40%	--------	(Carballa et al. 2005)
Conventional active sludge (CAS) treatment	anoxic pre-denitrification and phosphate precipitation with ferric chloride as tertiary treatment	65%	About 15% in 65% of total removal was through adsorption on to sludge	(Larsson et al. 2013)
Conventional biological wastewater treatment	bio filtration type wastewater treatment and consists of 30 bio filters	75%	Some percent of removal was occurred through adsorption onto sludge	(Lonappan et al. 2016)
Activated sludge process	--------	25%	--------	(Martín et al. 2012)
MBR	SRT(d)- 10-55 HRT(d) -0.5-4	<50%	--------	(Clara et al. 2005)

Conventional biological wastewater treatment	WWTP :1 SRT- 8days HRT- 9h WWTP : 2 SRT-18days HRT-23h	75% 39%	Study on 2 WWTPs. accumulation on sludge was of minor	(Samaras et al. 2013)
Conventional activated sludge with chemical phosphorous removal	HRT (h) 15-16	22%	---------	(Bendz et al. 2005)
conventional activated sludge with UV treatment as tertiary treatment	-------	81.4 %	---------	(Behera et al. 2011)
Conventional activated sludge with maturation pond Conventional activated sludge with chlorination Aerated lagoon with filtration and with chlorine addition	WWTP :1 SRT- 10days HRT- 6h WWTP : 2 SRT-6days HRT-6h	≈70% in all the 3 WWTPs	Study of 3 WWTPs.	(Anumol et al. 2016)

	WWTP : 2 SRT-3.1days HRT-74h			
Activated sludge	-----	45.6%	Average result for 15 different WWTPs	(Pereira et al. 2015)

Abbreviations - MBR: Membrane bioreactor, HRT: Hydraulic retention Time, SRT: Sludge retention time, MLSS: Mixed liquor suspended solids, WWTP: Wastewater treatment plant

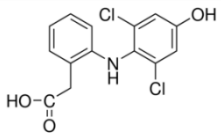
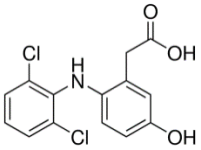
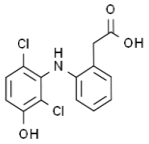
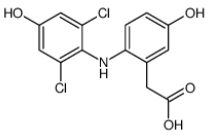
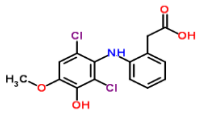
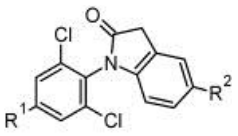
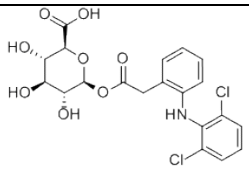
Table 3: Recent occurrences of diclofenac in aquatic environment

Environmental medium	Concentration ($ng\ L^{-1}$)	Country	Reference
River	2-3	Finland	(Lindqvist et al. 2005)
River	21-90	Canada	(Sosiak and Hebben 2005)
River	18-50	Canada	(Metcalfe et al. 2003)
Estuary	195	UK	(Thomas and Hilton 2004)
River	6.2	Germany	(Weigel et al. 2002)
River	1030	Germany	(Heberer 2002a)
Ground water/wells	2	Mediterranean region	(Rabiet et al. 2006)
Wells	380	Germany	(Heberer et al. 1998)
Drinking water tap	10	Germany	(Heberer 2002b)
River	100-200	Germany	(Letzel et al. 2009)
River	100-4900	Pakistan	(Scheurell et al. 2009)
Lake	370	Switzerland	(Buser et al. 1998)
River	5-40	UK	(Kasprzyk-Hordern et al. 2008)
River	26-72	Spain, Belgium, Germany, Slovenia	(Hernando et al. 2006)

River	20-91	UK	(Hilton and Thomas 2003)
Rivers and lakes	1.1-6.8	South Korea	(Kim et al. 2007)
River	0.7	France	(Rabiet et al. 2006)
Well	0.9	France	(Rabiet et al. 2006)
River	9-282	Slovenia	(Kosjek et al. 2005)
River	20-150	Switzerland	(Öllers et al. 2001)
Well	4.9-24	European Union (23 countries)	(Loos et al. 2010)
Aquifer	1.7	Spain	(Lopez-Serna et al. 2013)
Well	3.1	Spain	(Lopez-Serna et al. 2013)
River delta	29.5-380	Spain	(Lopez-Serna et al. 2013)
Drinking water	1.2	US	(Benotti et al. 2008)
River	15.8-35.5	Austria	(Ahrer et al. 2001)
River	7.8-64.8	China	(Dai et al. 2015)
Harbor lagoon	100	Pakistan	(Scheurell et al. 2009)
Well	590 (max. observed)	Germany	(Sacher et al. 2001)
River	260 (max. observed)	Spain	(López-Serna et al. 2012)

River	15	South Korea	(Yoon et al. 2010)
River	49	Spain	(Carmona et al. 2014)
Tap water	18	Spain	(Carmona et al. 2014)
River	34-145	Argentina	(Valdés et al. 2014)
Seawater (subtropical coastal zone)	19.4	Brazil	(Pereira et al. 2016)
River	230 (max. observed)	China	(Ma et al. 2016)

Table 4: Human metabolites of diclofenac

Metabolite	Molecular structure	Reference
4'-hydroxydiclofenac		(Boettcher et al. 1991; Stierlin et al. 1979; Stulten et al. 2008a)
5'-hydroxydiclofenac		(Boettcher et al. 1991; Stierlin et al. 1979; Stulten et al. 2008a)
3'-hydroxydiclofenac		(Boettcher et al. 1991; Stierlin et al. 1979)
4',5-dihydroxydiclofenac		(Boettcher et al. 1991; Stierlin et al. 1979)
3'-hydroxy-4'-methoxy diclofenac		(Boettcher et al. 1991)
4'-hydroxy diclofenac dehydrate		(Stulten et al. 2008a)
1-O-acyl glucuronide (DCF-gluc)		(Willis et al. 1979)

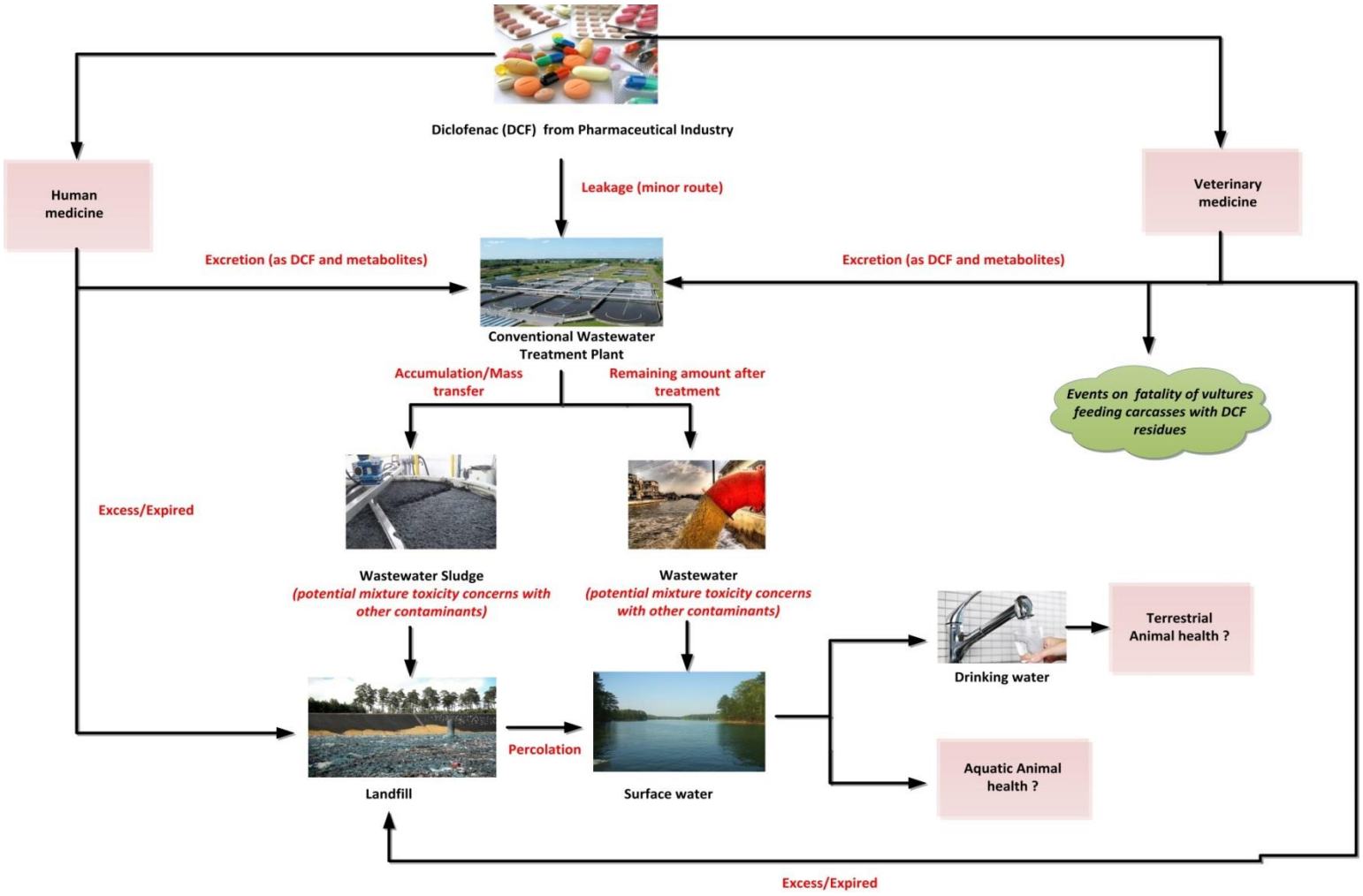


Figure 1: Entry routes of diclofenac to the environment

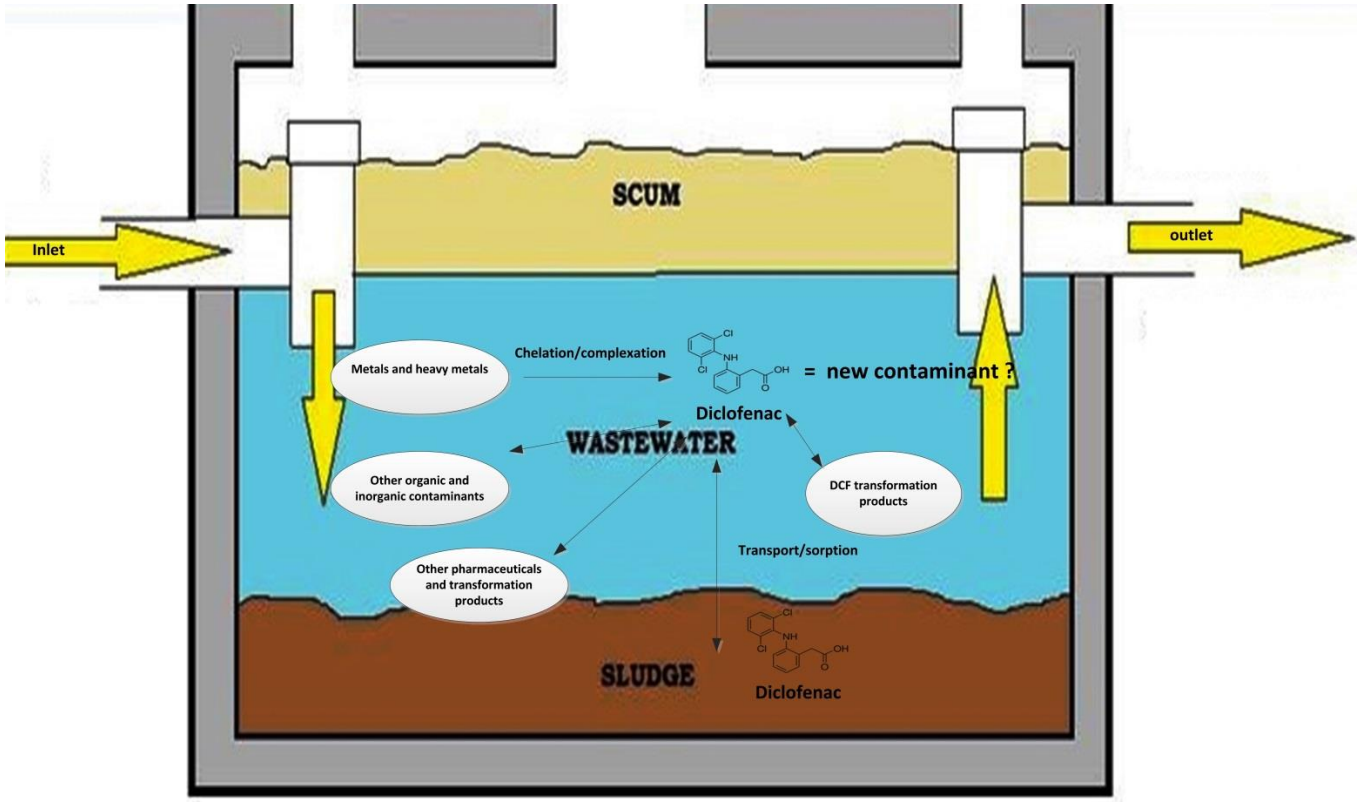


Figure 2: Hypothetical representation of a typical secondary treatment system in wastewater treatment plant showing interaction of diclofenac with other pollutants

PART 2

DICLOFENAC IN MUNICIPAL WASTEWATER TREATMENT PLANT: QUANTIFICATION USING LASER DIODE THERMAL DESORPTION-ATMOSPHERIC PRESSURE CHEMICAL IONIZATION- TANDEM MASS SPECTROMETRY APPROACH IN COMPARISON WITH AN ESTABLISHED LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION-TANDEM MASS SPECTROMETRY METHOD

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RÉSUMÉ

Le diclofenac, un anti-inflammatoire non-stéroïdien répandu, est souvent détecté dans les eaux usées et les eaux de surface. L'analyse de composés pharmaceutique dans les matrices complexes représente souvent un défi. Au cours de cette étude, une méthode fiable, rapide et sensible, basé sur l'utilisation de la désorption thermique par diode laser/ ionisation chimique à pression atmosphérique (LDTD/APCI-MS/MS) couplée avec la spectroscopie de masse en tandem (MS/MS), a été développé pour la quantification du DCF dans les eaux usées et les boues d'épuration. La méthode conventionnelle par LC-ESI-MS/MS (chromatographie liquide - ionisation par électronébulisation - spectroscopie de masse en tandem) a été comparé à l'approche par LDTD/APCI-MS/MS. La nouvelle méthode par LDTD/APCI-MS/MS permet d'effectuer l'analyse en 12 secondes par échantillon comparativement à 12 minutes pour la méthode par LC-ESI-MS/MS. Les limites analytiques de la méthode par LDTD/APCI-MS/MS ont été évaluées à 270 ng L-1 (LOD) and 1000 ng L-1 (LOQ). De plus, deux méthodes d'extraction du DCF à partir des boues d'épurations, extraction assistée par des ultrasons (EAU) et extraction par solvant accélérée (ESA), ont été comparées. L'ESA est la méthode la plus efficace avec 95.6 ± 7% de récupération en comparaison à 86 ± 4% pour l'EAU. Le destin et la distribution du DCF dans les eaux usées municipales et dans les boues d'épuration ont aussi été surveillés à différentes étapes de la station d'épuration des eaux usées de la Ville de Québec (Canada). Le DCF a présenté une plus importante affinité envers l'eau usée avec 60% de la quantité totale présente. Cette observation est contraire à la prédiction théorique (Log Kow= 4.51).

Mots clés : Diclofenac; contaminant émergent; eau usée, boue d'épuration; LC-ESI-MS/MS; LDTD-APCI-MS/MS; quantification

ABSTRACT

Diclofenac (DCF), a prevalent non-steroidal anti-inflammatory drug (NSAID) is often detected in wastewater and surface water. Analysis of the pharmaceuticals in complex matrices is often laden with challenges. In this study a reliable, rapid and sensitive method based on laser diode thermal desorption/atmospheric pressure chemical ionization (LDTD/APCI) coupled with tandem mass spectrometry (MS/MS) has been developed for the quantification of DCF in wastewater and wastewater sludge. An established conventional LC-ESI-MS/MS (Liquid chromatography-electrospray ionization- tandem mass spectrometry) method was compared with LDTD-APCI-MS/MS approach. The newly developed LDTD-APCI-MS/MS method reduced the analysis time to 12 seconds compared with 12 minutes for LC-ESI-MS/MS method. The method detection limits for LDTD-APCI-MS/MS method were found to be 270 ng L^{-1} (LOD) and 1000 ng L^{-1} (LOQ). Furthermore, two extraction procedures, ultrasonic assisted extraction (USE) and accelerated solvent extraction (ASE) for the extraction of DCF from wastewater sludge were compared and ASE with 95.6 ±7% recovery was effective over USE with 86±4 % recovery. The fate and partitioning of DCF in wastewater (WW) and wastewater sludge (WWS) in wastewater treatment plant was also monitored at various stages of treatment in Quebec Urban community wastewater treatment plant. DCF exhibited affinity towards WW than WWS with a presence about 60% of DCF in WW in contrary with theoretical prediction (Log K_{ow} = 4.51).

Keywords: Diclofenac- emerging contaminant, Wastewater, Wastewater sludge, LC-ESI-MS/MS, LDTD-APCI-MS/MS, Quantification

1. Introduction

Pharmaceuticals are a class of emerging contaminants which have recently raised great public concern due to its frequent occurrence in aquatic environment [1-3]. The consumption of these diverse classes of chemicals is expected to rise with augmenting population trends and consumption behavior of pharmaceuticals. Among pharmaceuticals, non-steroidal anti-inflammatory drugs (NSAID) are widely used throughout the world and detected in different environmental compartments at various concentrations ranging from ng L^{-1} to high µg L^{-1} [2, 4] since NSAID are over-the-counter (OTC) drugs. In the global scenario most popular pain killer, diclofenac (DCF) is also the most commonly used NSAID, with a market share close to that of the next three most popular drugs combined (ibuprofen, mefenamic acid, naproxen) [5]. Moreover, diclofenac can also be used as an anti-inflammatory agent, antiuricosurics and analgesic. A 2012 report from “Fierce Pharma” listed diclofenac as the 12^{th} bestselling generic molecule globally in a list topped by paracetamol. The total sales of diclofenac in 2011 was estimated to be $1.61 billion with an annual sales change of 15.5% [6]. Moreover, diclofenac is included in the emergency medical list (EML) of 74 countries. From Intercontinental Marketing Services (IMS) health data, it is estimated that about 940 tons of diclofenac is consumed globally on an annual basis [7]. This information proves that diclofenac is largely consumed throughout the world. Table 1 presents the physico-chemical and pharmacological properties of DCF.

Apart from the large volume consumption, DCF raises questions regarding environmental pollution. DCF is not completely removed from wastewater treatment plants (WWTP) due to its poor degradation and higher consumption rates which creates a continuous flow of the drug to the WWTP [8, 9]. Hence, DCF was detected in surface waters, sediments and sludges [10, 11]. Moreover, for the past several years, DCF has been an environmental concern due to its presence in drinking water sources [11, 12] and its potential harmful effects on many organisms at a concentration greater than 500 µg L^{-1} [13, 14]. Diclofenac has been to have potential toxicity towards aquatic life in surface waters in risk assessment studies [15] and has been predicted to be a major risk at higher

μg L^{-1} concentrations [16]. Moreover, DCF was the first widely noted case of a pharmaceutical causing major ecological damage [14] towards several vulture species in South Asia[17, 18].

Hence, it is important to remove the "potential" contaminant from its primary sink that is the wastewater treatment plant itself. Therefore, studies must be carried out for the quantification of DCF in the WWTP. The existing methods for the quantification of DCF are based on LC (liquid chromatography), UFLC (Ultra-Fast Liquid Chromatography) and HPLC (High-performance liquid chromatography). The major flaws of these methods are the prolonged time for analysis and matrix interferences. In the present study, a new and rapid method was developed based on LDTD-APCI-MS/MS for the quantification of DCF in WW and WWS and to the best of our knowledge, this study reported rapid method for the quantification of DCF in wastewater matrix. The newly developed rapid method was collated with an existing established LC-ESI-MS/MS method. Accelerated solvent extraction (ASE) and ultrasonic extraction (USE) was studied using spiked recovery experiments for the extraction of DCF from WWS using the newly developed LDTD approach and conventional established LC approach. In addition, the fate and partitioning of DCF in WW and WWS in WWTP was monitored at various stages of treatment in Quebec Urban community (CUQ) wastewater treatment plant. To the extent of our knowledge, partitioning study of DCF at various stages of a WWTP had never been carried out and this is the first study of its kind.

2. Materials and methods

2.1. Reagents

Diclofenac sodium salt (98%; CAS 15307-79-6), HPLC-grade methanol (MeOH, purity > 99.8%), HPLC grade acetonitrile (ACN, assay 99.9%), glacial acetic acid (assay: ≥99.7% w/w) and ammonium hydroxide (NH_4OH, 28–30% w/w) were purchased from Fisher Scientific (Ottawa, ON, Canada). Internal standard (IS), diclofenac-d_4 was obtained from C/D/N isotopes Inc. (Montreal, QC, Canada). Sep-Pak Plus C18 environmental cartridges used for solid phase extraction (SPE) and clean-up was supplied by Waters (Milford, MA, USA). Disodium ethylenediaminetetraacetic acid

(Na_2H_2EDTA, 99%) was purchased from E-bay (Tokyo, Japan). Chromatography grade water was prepared in the laboratory using Milli-Q/Milli-RO Millipore system (Milford, MA ,USA). Stock solutions of diclofenac and diclofenac-d_4 (1g L^{-1}) were prepared in methanol and stored in amber coloured bottles at 4±1 º C, until use. Working standards were prepared on the days of analysis by diluting stock solutions in acetonitrile.

2.2 Wastewater treatment plant, sampling and sample storage

The wastewater treatment plants in Quebec serve 7 million people and the network constitutes 702 treatment plants. These plants handle approximately 6.6 million m^3 wastewater per day. Wastewater and wastewater sludge samples were collected from eastern station of Quebec urban community (CUQ) wastewater treatment plant located in Quebec City, Canada. The CUQ plant receives wastewater from different sources of both domestic and industrial origin. The plant also receives wastewater from different hospitals and commercial enterprises spread all over the city. The eastern plant receives approximately 150,000 m^3 of wastewater per day and has a treatment capacity of 400,000 $m^3\,day^{-1}$. The eastern plant belongs to biofiltration type wastewater treatment and consists of 30 biofilters , 4 mechanical rake-cleaned bar screens, 5 aerated grit chambers, 7 laminar clarifiers and 5 disinfecting channels with 8480 ultraviolet lamps.

Wastewater and sludge samples were collected as grab samples in February 2015. All the samples were collected in triplicates and each sample was analyzed in duplicate. The collected samples were influents from sewage network system, wastewater after grit removal, primary wastewater and primary sludge, secondary wastewater and secondary sludge and the final effluent from the WWTP. Samples were collected in pre-cleaned high-density polyethylene (HDPE) containers. The samples were stored under dark conditions at 4±1 º C, until preparation for analysis which occurred within one week after the sampling.

2.3 Sample preparation

The liquid fraction of the WWS (both primary and secondary) was separated from the WWS by centrifugation at 7650 *x g* for 20 minutes. WWS and the solid fraction of the WWS samples were frozen at -20 º C for 24 hours and later lyophilized using freeze-dry system (Scanvac CoolSafe 55-4, Labogene, Lynge, Denmark). The solid obtained was gently ground using a mortar and pestle and stored at 4±1ºC until analysis. Wastewater samples (influent, primary wastewater, secondary wastewater, primary WWS-liquid fraction, secondary WWS-liquid fraction and the effluent) were filtered through a 0.45 µm glass-fiber (G6 filter circles, Fisher brand) using filtration unit and stored at 4±1 º C in amber coloured glass bottles, until clean-up process.

2.4 Extraction

Accelerated solvent extraction (ASE) and ultrasonic extraction (USE) were utilized to study the extraction efficiencies of the methods and to obtain a maximum recovery of DCF from WWS. About 0.5 g of sludge sample was optimized for the extraction and methanol was used as the solvent. In order to study the extraction efficiency, WWS samples were spiked with known concentrations of DCF at 10 µg g^{-1} and 1 µg g^{-1} before extraction.

2.4.1 Ultrasonic extraction (USE)

Ultrasonic extraction was carried out in an ultrasonic water bath (Fisher, FB 15069), which is preset to produce a frequency of 37 kHz. About 0.5 g of sludge was transferred into a 50 ml glass tube followed by the addition of 20 ml of methanol and shaken for 15 minutes in a vortex mixer at 1500 rpm. Sonication was employed for 15 minutes at 30±1 º C followed by degasing for 3 minutes. The extract was separated by centrifugation at 37 000 *x g* for 20 minutes. The entire extraction procedure was repeated twice. The extracts were combined and concentrated to approximately 1 mL with gentle stream of nitrogen in a heating module evaporation unit (Reacti-Therm I, Fisher Scientific, Canada) at 35 ±1 º C. Finally, the extracts were re-dissolved in 50 mL of chromatography grade

water and then subjected to solid phase extraction and clean-up process as discussed in section 2.5.

2.4.2 Accelerated solvent extraction (ASE)

Accelerated solvent extraction was carried out using Dionex ASE 350 accelerated solvent extractor (Dionex, Sunnyvale, CA, USA). About 0.5 g of lyophilized and homogenized sludge sample was extracted in a 34 mL stainless steel cell. The optimized extraction process was performed at 100 ± 1^{0} C and at 1500 psi for 10 minutes heat-up mode followed by 5 minutes of static extraction with solvent saver mode. The extraction process was completed by 1 minute of nitrogen purge step. Subsequently, the extracts were concentrated to approximately 1 mL using gentle stream of nitrogen in a heating module evaporation unit (Reacti-Therm I, Fisher Scientific, Canada) at 35 ±1 ºC. Finally, the extracts were re-dissolved in 50 mL of chromatography grade water and subjected to solid phase extraction for clean-up as discussed in section 2.5.

2.5 Solid phase extraction

Solid phase extraction was employed for concentrating and cleaning up the extract obtained from USE and ASE methods and also for wastewater samples. Sep-Pak C18 plus Short Cartridges (360 mg sorbent per cartridge, surface area 313 (m^2 g^{-1})) were used for extraction. Cartridges were fitted into vacuum manifold (Welch, USA) which was connected to a vacuum pump (Welch Rietschle Thomas, USA) for homogeneous extraction. The extraction process was started with pre-conditioning of cartridges using 6 mL of methanol followed by 6 mL of chromatography grade water at a flow rate of 2 mL min^{-1}. The filtered samples were loaded to the cartridges and extracted at a flow rate of approximately 3 mL min^{-1}. After the extraction, the sorbents were dried at 15 psi in the vacuum manifold for 15 minutes. The elution was performed by 3 x 4 mL of methanol/acetonitrile mixture (50:50, v/v) solution at an approximate flow rate of 2 mL min^{-1}. Later, the extracts were evaporated to dryness under gentle stream of nitrogen and reconstituted to 1 mL with acetonitrile. The extracts were then analyzed using LDTD-MS/MS and LC-MS/MS.

2.6 LDTD-APCI-MS/MS analysis

Quantification of DCF in WW and WWS samples was achieved by newly developed LDTD-APCI-MS/MS method. The instrument comprises LDTD–APCI (atmospheric pressure chemical ionization) source (LDTD T-960, Phytronix Technologies, Quebec, Canada) mounted on a TSQ Quantum access triple quadruple mass spectrometer (Thermo Scientific, Mississauga, Ontario, Canada). The LDTD–APCI source was fitted with a compartment for bar coded LazWell TM plate (96-well plate) to introduce liquid samples for analysis. Before spluttering the samples on the bottom of stainless steel alloy based LazWell plate; samples were mixed with EDTA (0.1 mg/mL, prepared in a mixture of methanol, water and ammonium hydroxide) solution for complete thermal desorption of DCF from the LaZwell plate. About 4 µL of sample was spluttered carefully on to the well of the plate and left to dry at 35±1 º C in an oven. The LazWell plate with dried samples was then transferred to an X–Y movable stage of the LDTD sample compartment for LazWell plates. Thermal desorption was achieved by infrared (IR) laser diode (980 nm, 20 W, continuous) which was focused on the back of the plate of each wells. Carrier gas (compressed air stream, medical grade) carried the desorbed sample through a glass tube which will carry the analyte to the corona discharge region where the analyte was ionized by APCI. The ions were then introduced directly to the MS for analysis. Quantification was carried out by internal standard method using diclofenac-d_4 (10 µg L^{-1}) as internal standard. Standards were prepared in acetonitrile supplemented with EDTA and 10 µg L^{-1} of internal standard. The area ratio was plotted against the analyte concentration ratio to obtain the calibration curve and excellent linearity was obtained with R^2 value always greater than 0.990.

Temperature and kinetics of the thermal desorption was controlled by laser power and which was optimized for optimal signal for the analysis. The laser power was ramped from 0 to 65 % in 8 s, and maintained for 2 s. The APCI was carried out with a positive corona discharge, current of 3 µA, a carrier gas temperature of 30±1 ºC and an air flow rate of 3 L min^{-1}. The mass spectrometer system was operated in single reaction monitoring (SRM) mode. The optimized method resulted in rapid

analysis of sample in 12 s for LDTD process from sample to sample with no traces of carry-over. The optimization was tested with four consecutive analytical replicates.

2.7 LC-ESI-MS/MS analysis

DCF in WW and WWS samples was also analyzed using LC-ESI-MS/MS method (Thermofisher scientific, application Note 20569, www.thermoscientific.com). The LC-MS/MS system was composed of Finnigan surveyor LC pump equipped with a 120-vial sample management system. The LC pump was coupled with a TSQ Quantum access triple quadruple mass spectrometer (Thermo Scientific, Mississauga, Ontario, Canada) and an electrospray ionization interface. The analysis was carried out using 'BetaBasic C18 LC column' (3 µm,100 x 2.1mm,Thermo Scientific, Peterborough, ON, Canada).

The LC-MS/MS method used in this study is a modified method developed from thermo fisher scientific LC-MS/MS method for the determination of diclofenac in human plasma (Application Note 20569). For LC-MS/MS analysis standards, internal standard and samples were prepared in chromatography grade water. About 500 µL of sample was prepared in amber coloured HPLC vial. An amount of 1 mg L^{-1} of internal standard (diclofenac-d4) was used to get maximum signal intensity for all samples. The mobile phase used was the mixture of water and 0.1% acetic acid (mobile phase A) and acetonitrile and 0.1% acetic acid (mobile phase B). Other parameters comprised: flow rate - 0.5 mL min^{-1}, column temperature - 35±1 ºC and an injection volume of 20 µL. Electrospray ionization (ESI) was set with a negative polarity and the collision gas pressure was optimized to be 1.4 mTorr. The mass spectrometer system was operated in a single reaction monitoring (SRM) mode. The optimized method resulted in analysis time of 12 minutes from sample to sample with no traces of carry-over.

2.8 Data and statistical analysis

Recovery experiments were carried out in duplicates with both primary and secondary sludge. The recovery was calculated using equation (1)

$$recovery\ (\%) = {C_f - C_i}/{C_{sp}} \qquad (1)$$

Where, C_f – final measured concentration of DCF in the spiked matrix, C_i - initial concentration of DCF in the sludge matrix and C_{sp} – actual calculated concentration of DCF spiked in the matrix.

Limit of detection (LOD) was calculated from the standard error of the intercept and the slope of the calibration curve. The intra-day method repeatability (presented in % relative standard deviation) was calculated with replicate analysis of secondary sludge sample spiked with DCF. Inter-day precision (presented in % relative standard deviation) was calculated by freshly prepared sample of known concentration in wastewater matrix.

To investigate the statistical significance of the measured values of the standard curve one way ANOVA test has been carried out with 95% confidence level using Sigmaplot version 11.0 (Systat Software GmbH, Germany). It was observed that there is no statistically significant difference ($p < 0.05$) between the same concentrations in the standard curve.

3. Results and Discussion

3.1 Quality assurance

Standards were analyzed 4 times and the relative standard deviation (RSD) was below 10% for LDTD and LC method. Correlation coefficient (R^2) obtained from standard curves of the replicates were always greater than 0.990 for both methods. Use of internal standard, diclofenac-d_4 further enhanced the accuracy and precision. Limit of detection (LOD) and Limit of Quantification (LOQ) was also calculated.

Internal calibration was carried out using internal standard DCF-d4. The precision and accuracy of the methods were further studied using recovery experiments by spiking wastewater and sludge samples with internal standard. Internal standard spiking was carried out using 10 µg L^{-1} of DCF-d4 before ASE. In addition, another set of experiments with wastewater influent samples and ASE extracts were also carried out by spiking with 10 µg L^{-1} of DCF-d4 after extraction. The internal standard area was monitored in both cases and no significant change ($p < 0.05$) in internal standard concentration was observed.

3.2 LDTD –APCI –MS/MS method

Ultrafast quantification with high precision and accuracy was obtained using LDTD-MS/MS method with APCI ionization. The peak with best intensity was obtained in APCI positive mode. MS/MS scan was operated through available SRM mode in TSQ Quantum access triple quadruple mass spectrometer. Sample volumes between 8 µL to 2 µL were tested and optimized to 4 µL for a sharp peak. Other LDTD parameters, such as sample volume, laser power, carrier gas flow and laser gradient were optimized which affect the analysis performance, such as sensitivity, reproducibility, peak area and shape. For complete desorption of DCF from LaZwell plate EDTA (0.1 mg mL^{-1}, prepared in a mixture of methanol, water and ammonium hydroxide) was added to all samples and standards. In LDTD-APCI-MS/MS, LASER beam desorbs the compound from LaZwell plate and which is ionized by APCI and transferred to MS/MS. LDTD is based on the principle of thermal desorption of compounds from LaZwell plate by LASER beams. Hence, the LOQ and MDL of compound depends on desorption capability of the compound from the LaZwell plate by LASER. Compounds having carboxylic acid and chlorine atoms in their structures, such as DCF and chlortetracycline [19] have shown higher LOQ with LDTD. Hence, for these classes of compounds, EDTA has been used for the complete desorption of the compound from the plate. Methanol and acetonitrile were tested as solvents to be used for analyte deposition on cavities in the sample well. Maximum peak intensity was achieved with acentonitrile and hence acetonitrile was assigned to be the preferred solvent for analyte deposition on the wells of LaZwell plate. The optimization of the

method was carried out following the previously reported studies for pharmaceuticals in WW and WWS. C18 cartridges showed excellent recovery for diclofenac from previously reported studies [20]. Hence, sep-pak C18 cartridges were chosen for SPE. For quantification and method validation, LDTD-MS/MS optimized parameters were applied. Several calibration curves were prepared with a 7 point (1- 100 µ L^{-1}) calibration curve and a good linearity was observed (R^2 > 0.990). Based on signal-to-noise ratio of calibration, curve limits of detection (LOD) and limit of quantitation (LOQ) were calculated. Intra-day repeatability and inter-day reproducibility are expressed as relative standard deviation (% RSD) and was found to be 7.1 and 9.2, respectively. Hence, it is evident that LDTD approach exhibited excellent sensitivity for DCF throughout the day. Limit of detection (LOD) and limit of quantification (LOQ) was obtained as 270 ng L^{-1} and 1000 ng L^{-1}, respectively. The method detection limit (MDL) was calculated to be 300 ng L^{-1} for wastewater samples and 75 ng g^{-1} for wastewater sludge samples. The experiment was carried out using 10 replicates and repeatability and reproducibility (% RSD) was always observed to be less than 10 % throughout these repeated experiments. For wastewater samples, the observed values were 7.3 % and 9.1 %, respectively for repeatability and reproducibility and for wastewater sludge samples, it was 8.6 % and 9.8 %, respectively. Previously reported studies in wastewater matrix exhibited LOQ values over a range from 1 µg L^{-1} to 50 µg L^{-1} [21-24] and the present study also achieved an LOQ of 1 µg L^{-1}.

3.3 Comparison of measured DCF levels in WW and WWS using an established LC-ESI MS/MS method versus LDTD-APCI -MS/MS approach

LC-ESI MS/MS method used for this study was an established method for the determination of DCF in human plasma (Thermofisher scientific, application Note 20569, www.thermoscientific.com) Further optimization of the method was carried out since the instrument used in this study are not exactly same as the established method. In wastewater matrix, this optimized method achieved a LOQ of 1 µ L^{-1} which is exactly same as that obtained for LDTD method. Several calibration curves were prepared with a 7 point (1- 100µ L^{-1}) calibration to check the precision and accuracy of the method and a good linearity was observed (R^2 > 0.990).

Both LDTD and LC methods exhibited excellent ability for the analysis of DCF in WW. Chromatograms for both of the methods with WW and WWS are given in Figure 1 and Figure 2. From Figure 1(a) and Figure 2 (a), it is clear that for wastewater, matrix effect was negligible in both LC and LDTD approaches. Both the methods showed excellent agreement (< 10%) in analytical results for the quantification of DCF in WW. The difference in analytical values lies between 95 to 105 % for WW samples. Even though for WWS, LDTD exhibited advantages over LC method. There was not a good correlation between the values obtained for the concentration of DCF in WWS using LDTD-APCI-MS/MS method and LC-ESI-MS/MS method. The observed concentrations of DCF in WWS matrix using LC-ESI-MS/MS method lay between 65 % to 90 % as compared to the values observed with LDTD-APCI-MS/MS approach constituting an unacceptable difference of 35%. The values were confirmed to be true by spiking the sludge samples with known concentration of DCF and LDTD-APCI-MS/MS approach exhibited excellent agreement with the known spiked concentrations. Since the difference was compared with WWS which is high organic matrix environmental sample, the complexity of sample matrix in LC-MS/MS methods cannot be overruled [22, 25, 26]. Also, it has to be noted that signal suppression due to unknown complex matrix interferences is a common problem in quantitative analysis, especially in ESI which is used for the present study with LC-MS/MS [27, 28]. Matrix effects, caused by analyte and matrix component interactions, are unique to ESI-based LC- MS/MS approach and are one of the biggest challenges when working with LC-MS/MS. In this study, Figure 1 represents the matrix interference phenomenon. Figure 1(b) represents a noisy signal .The matrix effects can be classified into matrix interference and signal alteration [26, 28]. The suppression could be due to the interactions (such as, Van der Waals, dipole-dipole, dipole or electrostatic forces) between DCF and other co-extracted compounds present in the sample that could suppress ionization of a DCF in the ESI source. This must have resulted in a lower signal and ended up detecting false negative results. Further efforts to enhance the signal were not carried out since it was not the major aim of the study and a suitable method was already developed via LDTD-APCI-MS/MS approach. In LDTD-APCI-MS/MS, instead chromatographic separation laser desorption of the sample has been employed along with less

matrix sensitive APCI which further improved the sensitivity (Figures 2(a) and 2(b)).From these figures, it is evident that matrix interferences have not substantially affected the signal. Moreover, time for the analysis per sample was reduced from 12 minutes in LC-ESI-MS/MS to 12 seconds in LDTD-APCI-MS/MS. Furthermore, LDTD approach appeared to be sustainable and green since it omitted the use of chromatographic solvents.

Throughout this study, inconsistencies associated with manual spotting to LaZwell plate for the LDTD-APCI-MS/MS analysis was a major concern. The amount to be spotted to the LaZwell plate is too small (4 µL) and which makes the LDTD-APCI-MS/MS method sensitive to manual error. The rapidness in LDTD-APCI-MS/MS analysis has come with an expense. When comparing the cost of analysis per sample for LDTD-APCI-MS/MS analysis with conventional LC-MS/MS analysis LDTD approach appears to be more expensive particularly owing to the expense of LaZwell plate (approximately 100 USD per single disposable 96-well LaZwell plate) along with other expenses. Nevertheless, LDTD approach has its own advantage over other methods due to its ultrafast quantification.

3.4 Comparison of USE and ASE methods

A series of experiments has been carried out to find the best extractive procedure for the extraction of DCF from WWS. The extraction methods were evaluated and compared on the basis of recovery efficiency of DCF from WWS. The recovery experiments were performed by spiking both primary and secondary WWS using 10 µg g^{-1} and 1 µg g^{-1} of DCF. The extraction was carried out using methanol as solvent following the previous studies. The solvent with considerable relative affinity towards diclofenac has been chosen while reducing the dissolution of impurities and non-targeted compounds [29-31]. The recovery using USE was 74±3 % and 86±4 % for primary and secondary sludge, respectively. For ASE, the recovery obtained was 86±2 % for primary sludge and 95.6±7% for secondary sludge. The difference in recovery for primary and secondary sludge might be due to the difference in matrix and composition of the sludge. Primary sludge was composed of inorganic

matter and is fibrous in nature. This property gives better sorption strength and hence the extraction from primary sludge becomes difficult. On the contrary, secondary sludge was composed of organic matter. Even though, in general, organic matter is an effective adsorbent, yet desorption from organic matrix is also comparatively a rapid process. Hence, better recoveries were obtained with secondary sludge. The method recovery obtained by this study is in accordance with the recoveries for DCF reported by previous studies for sludge in general [24, 30-33]. ASE exhibited better extraction efficiency over USE. The desorption of DCF from the active spots of the matrix is the rate limiting step in the extraction process. For ASE, the extraction was carried out at higher temperature (100±1ºC) and pressure (1500 psi) followed by additional nitrogen purging. The higher temperature and pressure lowered the surface tension and viscosity of the liquid and allowed better contact between DCF and solvent. Moreover, higher temperature improved the dissolving capacity and reduced the physical binding forces between analyte and binding spots in the matrix.

Recovery experiment was also carried out to study the solid phase extraction efficiency by C18 cartridges. Experiments were carried out using 5 replicates for wastewater samples (influent and effluent) at 3 different spiking levels (5 µg L^{-1}, 10 µg L^{-1} and 50 µg L^{-1}). Excellent recoveries were obtained ranging from 98.2 % to 104.6%. For spiking level of 5 µg L-1, a recovery of 100.4±2 % was obtained. For spiking level of 10 µg L-1, a recovery of 98.2 ± 3% was obtained and for spiking level of 50 µg L -1 recovery obtained was 104.6 ± 1%.

3.5 Application of optimized method for the fate study of DCF in WWTP

3.5.1 Partitioning of DCF in WWS

About 75 % removal was obtained with the conventional treatment system of the wastewater treatment plant. Nevertheless, when analyzing the concentration of DCF in sludge, it can be concluded that there is less degradation but accumulation in sludge. Both primary and secondary sludge samples comprised higher DCF concentration as shown in Figure 3. The K_{ow} values of DCF (Table 1) suggest accumulation of DCF in sludge phase and previous studies observed an opposite

phenomenon [8, 23, 24, 34]. This study is also in line with the previously reported studies. When analyzing the partitioning of DCF in WWS and WW, DCF exhibited affinity towards WW over WWS. The solids concentration was observed to be 10±0.5 g L^{-1} and 8±0.3 g L^{-1} for primary and secondary sludge, respectively. Hence, the removal/degradation at primary and secondary levels has been calculated. About 18% removal/degradation was observed at the grit removal state. Wastewater with about 52.75 µg L^{-1} of DCF has been transferred to the primary treatment system. About 20% of this concentration was accumulated in the sludge and 13 % degradation was observed and the rest of the DCF has been transferred to secondary treatment system. The secondary treatment system exhibited higher degradation that is 31% of the received concentration and the rest was accumulated in sludge and wastewater. Hence, apart from removal through sludge, an overall degradation of about 50 % was observed in the WWTP.

3.5.2 Occurrence of DCF in WWTP

The occurrence of DCF at various stages of wastewater treatment process has been presented in Figure 3. High concentration of DCF has been detected in CUQ wastewater treatment plant. The concentration detected in the influent was 64.89 µg L^{-1} . Higher concentration of DCF can be attributed to the consumption behavior of the drug. DCF is an over the counter drug along with its direct application to the skin as pain reliever such as volteran gel. Furthermore, DCF has been established as a veterinary medicine. Moreover, DCF is a drug which is associated with lifestyle diseases and in a developed country such as Canada where lifestyle diseases are prevalent, higher consumption of DCF can be expected. In addition, the Quebec Urban Community WWTP receives wastewater from different hospitals in the vicinity of the Quebec City area. This fact validates high detection of DCF in CUQ plant. The effluent from the wastewater treatment plant contains 15.95 µg L^{-1} of DCF. Overall, the plant accounted for about 75 % removal of DCF from wastewater during the treatment process. This removal efficiency was within the range of previously reported studies for the removal of DCF in WWTP [34]. The effluent contaminated with DCF was the result of the absence of UV treatment which is reported to be an effective method for the removal of DCF from

WW [35-37]. The CUQ plant employs the UV treatment system only during summer and the sampling was carried out in winter and hence for the same wastewater treatment plant better removal efficiency can be expected during summer period.

4. Conclusion

A novel rapid method for the quantification of diclofenac in wastewater and wastewater sludge has been developed by LDTD-APCI MS/MS approach. Method was highly sensitive with limit of quantitation of 1 µ L^{-1} and an ultrafast quantification time of 12 seconds. The LDTD-APCI-MS/MS method can be effectively employed for the detection and quantification of DCF in WW and WWS samples instead of traditional slow, matrix sensitive LC-ESI-MS/MS method. The LDTD approach replaces chromatographic separation by laser diode thermal desorption and hence avoid the use of organic solvent which is a leap towards green chemistry principles. Accelerated solvent extraction was found to be effective extraction method over ultrasonic extraction for the extraction of DCF from WWS. Higher concentration of 64.89 µg L^{-1} was detected in the wastewater influent. Accumulation of DCF in primary sludge (1.10±0.15 µg g^{-1}) as well as secondary sludge (0.90±0.15 µg g^{-1}) was observed along with the degradation of DCF in WWTP. Hence, both WW and WWS must be taken into account while developing treatment methods for DCF in a typical wastewater treatment plant.

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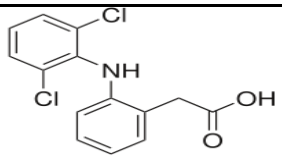
Table 1: Physico-chemical properties of diclofenac

Structure	
Molecular formula and molecular weight	$C_{14}H_{11}Cl_2NO_2$, 296.16 g mol^{-1}
Water solubility at 25 º C	2.37 mg L^{-1} (Diclofenac) 50 g L^{-1} (Diclofenac Sodium)
Melting and boiling points	283-285 ºC and 412 º C at 760 mm Hg (Predicted)
Henry's law constant	4.79×10^{-7} Pa m^3 mol^{-1} (25 º C)
Log K_{ow}	4.51
p*K*a	4.15
Mode of usage	Oral, rectal and direct application to skin
Applications	Both veterinary and human

References: syrres.com, www.sigmaaldrich.com, www.drugbank.ca, www.scbt.com

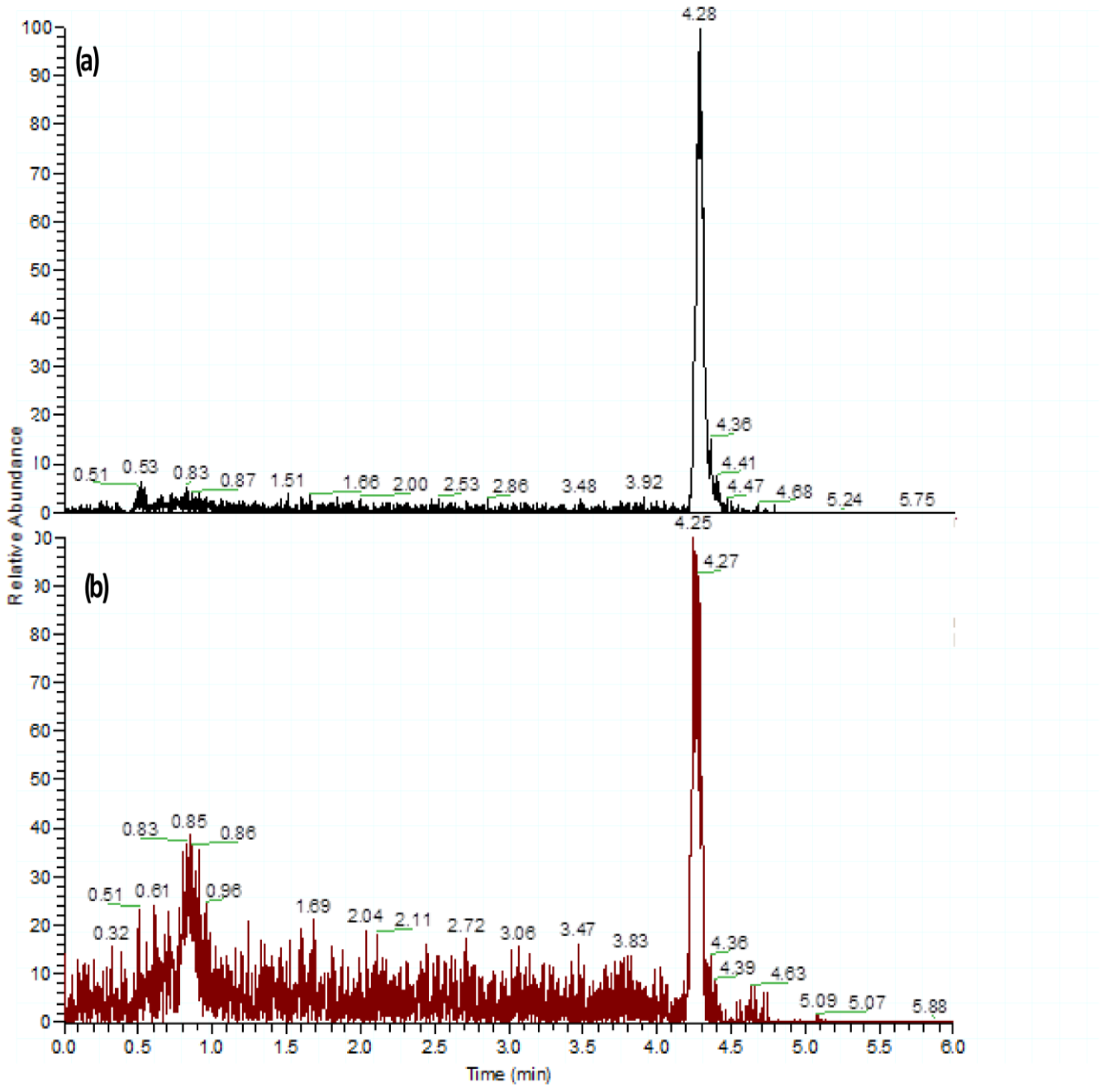


Figure 1: Chromatograms - representative peaks of diclofenac in: (a) wastewater with LC-ESI-MS/MS; (b) wastewater sludge with LC-ESI-MS/MS

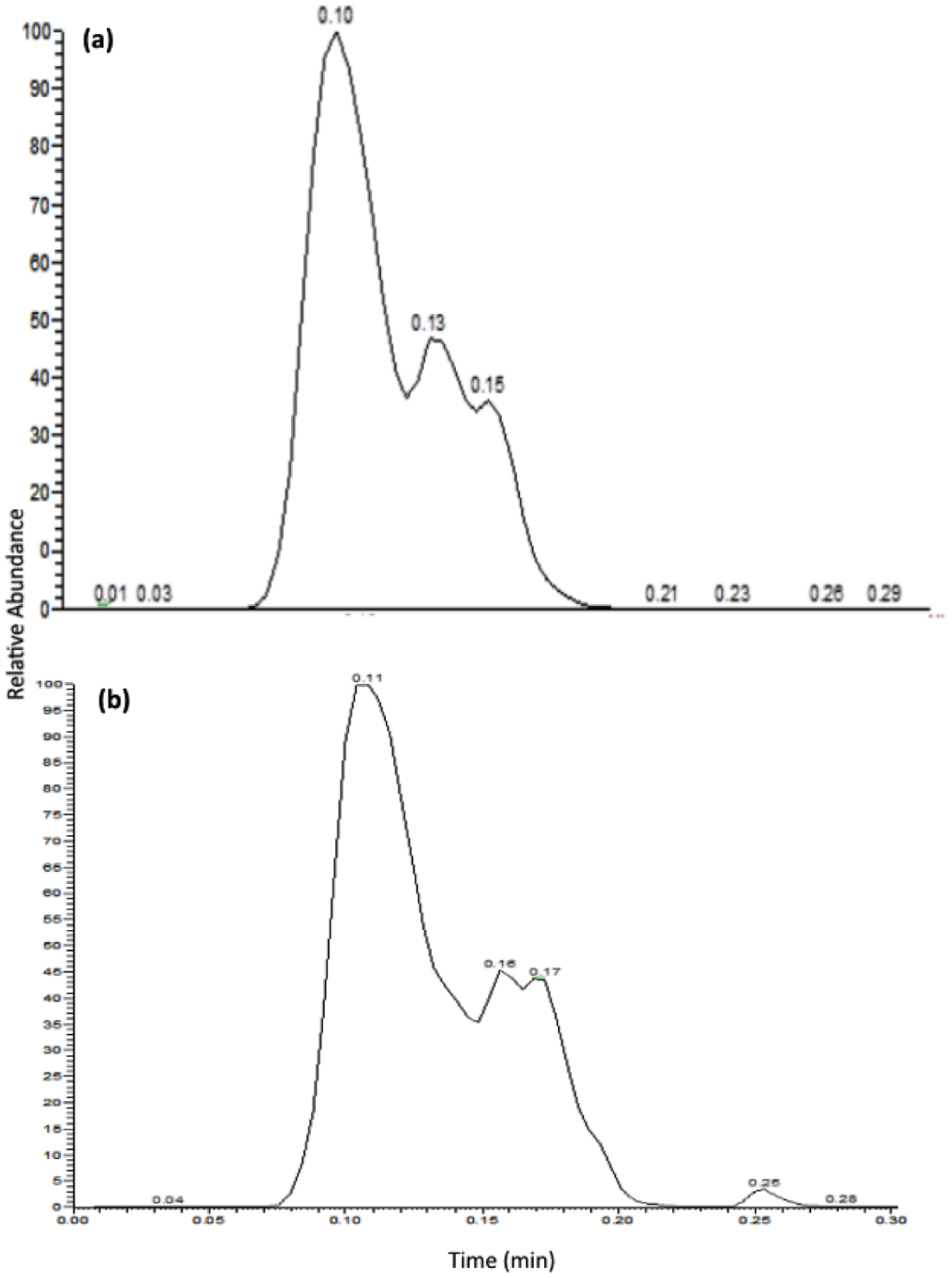


Figure 2: Chromatogram – representative peaks of diclofenac in: (a) wastewater with LDTD-APCI-MS/MS; (b) wastewater sludge with LDTD-APCI-MS/M

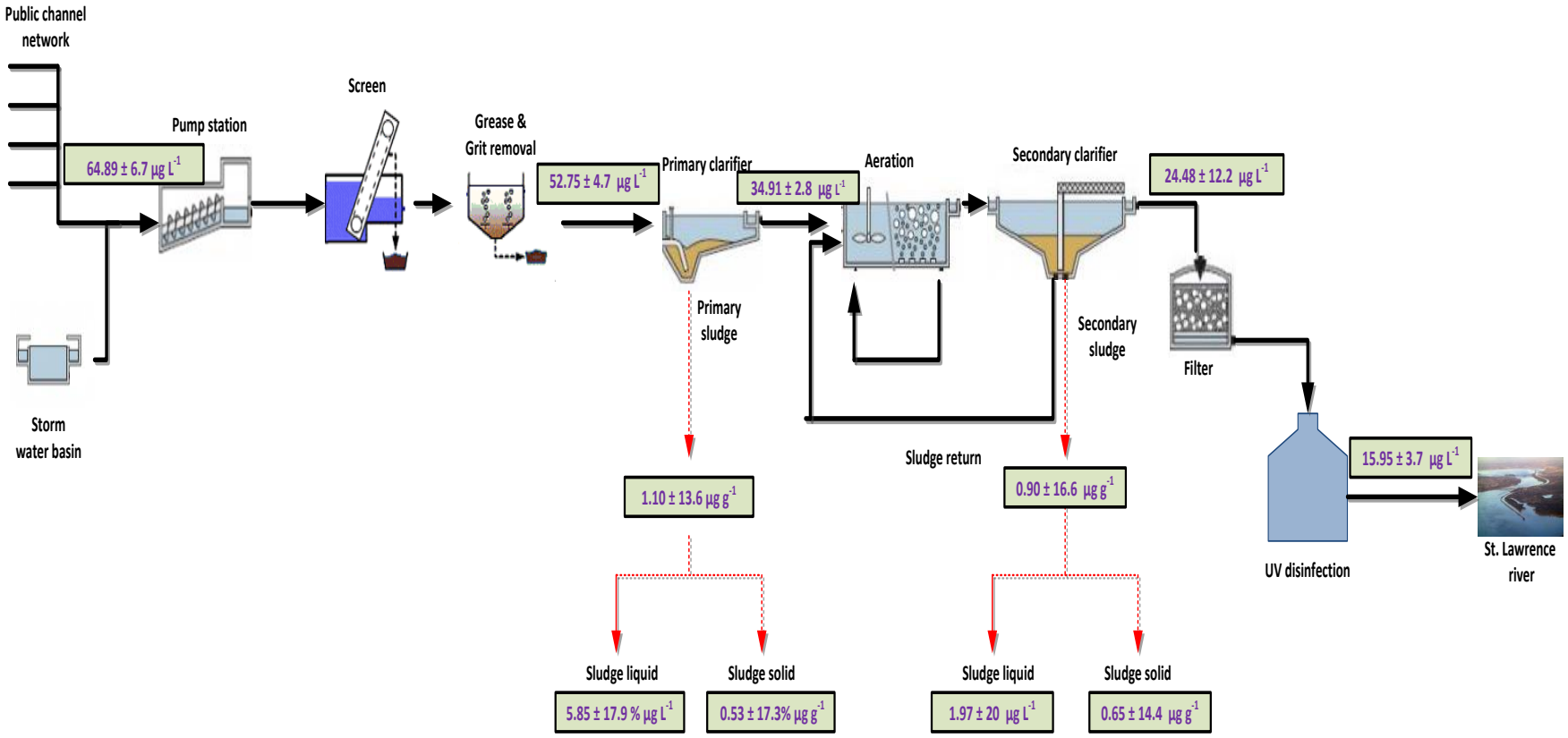


Figure 3: Fate and partitioning of diclofenac (with mean concentration of DCF (mean± SD, n=3)) in CUQ wastewater treatment plant

CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF MICRO-BIOCHARS AND ADSORPTION STUDIES FOR THE REMOVAL OF DICLOFENAC

PART 1

ADSORPTION OF METHYLENE BLUE ON BIOCHAR MICROPARTICLES DERIVED FROM DIFFERENT WASTE MATERIALS

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RÉSUMÉ

Les microparticules de biochar ont été préparées à partir de trois différents types de biochar (biochar de résidus de bois de pin (BC-PW), de fumier de cochon (BC-PM) et de carton (BC-PD)) sous différentes condition pyrolytiques. Les particules ont été préparées par broyage à sec et tamisage séquentiel sur différents tamis ASTM. La taille des particules et la surface spécifique ont été analysés à l'aide d'une analyseur de taille de particule au laser. Les particules ont ensuite été caractérisés par microscope à balayage électronique (SEM). La capacité d'adsorption de chacun des biochar a été déterminée par des test d'adsorption au bleu méthylène et comparée aux capacités des charbons activés commercialement disponibles. Les résultats expérimentaux démontrent que la capacité d'adsorber le colorant augmente avec la concentration du colorant et le dosage de biochar. Les microparticules de biochar préparées à partir de différents résidus ont manifestées une capacité d'adsorption supérieure (7.8±0.5 mg g^{-1} to 25±1.3 mg g^{-1}) aux biochar brut et aux charbons activés commercialement disponibles. La capacité d'adsorption du biochar varie selon le type de résidus utilisé et la méthode de production. La capacité d'adsorption maximale (25 mg g^{-1}) a été obtenue avec les microparticules de BC-PM à 25°C pour une concentration de colorant de 500 mg L^{-1} comparativement à 48.30±3.6 mg g^{-1} pour le charbon activé commercial. Les cinétiques d'adsorption pour les biochar BC-PM et BC-PD respectent le model de Langmuir alors que la cinétique d'adsorption pour le biochar BC-PW est mieux représentée par le model de Freundlich.

Mots clés : Biochar; Adsorption; Microparticules; Bleu de méthylène; résidu

ABSTRACT

Biochar microparticles were prepared from three different types of biochar, derived from waste materials, such as pine wood (BC-PW), pig manure (BC-PM) and cardboard (BC-PD) under various pyrolysis conditions. The microparticles were prepared by dry grinding and sequential sieving through various ASTM sieves. Particle size and specific surface area were analyzed using laser particle size analyzer. The particles were further characterized using scanning electron microscope (SEM). The adsorption capacity of each class of adsorbent was determined by methylene blue adsorption tests in comparison with commercially available activated carbon. Experimental results showed that dye adsorption increased with initial concentration of the adsorbate and biochar dosage. Biochar microparticles prepared from different sources exhibited improvement in adsorption capacity (7.8±0.5 mg g^{-1} to 25±1.3 mg g^{-1}) in comparison with raw biochar and commercially available activated carbon. The adsorption capacity varied with source material and method of production of biochar. The maximum adsorption capacity was 25 mg g^{-1} for BC-PM microparticles at 25 °C for an adsorbate concentration of 500 mg L^{-1} in comparison with 48.30±3.6 mg g^{-1} for activated carbon. The equilibrium adsorption data were best described by Langmuir model for BC-PM and BC-PD and Freundlich model for BC-PW.

Keywords: Biochar; Adsorption; Microparticles; Methylene blue; Waste material

1. Introduction

Disposal of solid waste materials is a cause for concern throughout the world. Recent developments in environmental technology focused on the use of sustainable materials and advanced management practices for waste materials such as production of value-added products from waste materials. Biochar is a carbon-rich solid obtained by the pyrolysis of organic material. The organic materials can be waste materials of municipal or agricultural origin. The unique and specific properties of biochar include large surface area, highly porous structure, enriched surface functional groups and mineral components. These unparalleled properties make biochar an effective material for mitigating global warming, soil amendment, enhancement of crop yield, carbon storage and removal of contaminants from water (Tan et al., 2015). Besides, biochar is being considered as a waste disposal and recycling option (Gupta et al., 2009). The specific properties of biochar, such as surface area and porous structure will depend upon the source material and method of production, such as pyrolysis temperature, thermochemical conversion technology and residence time (Tan et al., 2015).

Due to its economic feasibility and environmental relevance along with its physico-chemical properties, biochar can be used for the removal of contaminants. The adsorption potential of biochar is widely acknowledged with adsorption of wastewater pollutants, such as phenol (Tan et al., 2009), dye (Cheng et al., 2013) and heavy metals (Kołodyńska et al., 2012; Mohan et al., 2007). Relatively recently, biochar without any activation was identified as a 'supersorbent' for neutral organic compounds (NOCs) (Yang and Sheng, 2003). A copiousness of polar functional groups on biochar surface enhanced NOC adsorption by biochar compared to activated carbon (Yang et al., 2004). Thus, biochar offers good opportunities for removal of various organic contaminants.

The behaviour of microparticles is unique and quite different since microparticles have a larger surface-to-volume ratio than at the macro scale (Vert et al., 2012). According to International Union of Pure and Applied Chemistry (IUPAC) guidelines "Terminology for biorelated polymers and

applications (IUPAC Recommendations 2012)", microparticles are defined as particles with dimensions between 1×10^{-7} and 1×10^{-4} m, even though the lower limit between micro sizing and nanosizing is still a matter of debate since nanoparticles cover only the range of particle dimensions with 0.1 nm to 100 nm (Vert et al., 2012). This study followed the IUPAC guidelines for microparticles. Generally, biochar prepared via pyrolysis process are of larger dimensions, such as millimeters and micrometers depending upon the source material and method of production and are used as obtained from the process (Demirbas, 2004; Lei et al., 2009). The effort to classify micro particles according to size is uncommon except for nanoparticles (Kambo and Dutta, 2015; Yan et al., 2013). Hence, lacuna still exists in this field of research. Most of the previous studies were carried out on crude biochar (Banerjee et al., 2014; Shih, 2012; Yao et al., 2012). Maximum removal efficiencies for methylene blue observed in these studies were 4.58 mg g^{-1} for untreated saw dust biochar (Banerjee et al., 2014) , 8.07 mg g^{-1} for rice husk biochar (Shih, 2012) and 16.75 mg g^{-1} for pine sawdust biochar (Cheng et al., 2013). Few recent studies evaluated the efficiency of biochar nanoparticles in combination with other nanomaterials used for various applications, such as a catalyst (Saxena et al., 2014; Yan et al., 2013). No study data exists, to the best of our knowledge for biochar nanoparticles as a standalone adsorbent for methylene blue. In a recent study, magnetic Fe_3O_4 nanoparticles coated with sub-nano biochar exhibited excellent adsorption capacity of 349.40 mg g^{-1} for crystal violet (Sun et al., 2015). Crystal violet is a dye such as methylene blue, used for the adsorption capacity characterization of newly synthesized adsorbents. It is expected that nano biochar might exhibit excellent adsorption capacity over raw biochar or micro biochar because of its largest surface area; however production costs and fouling at times can limit the advantages. On the other hand with microparticles, an increase in adsorption potential can be expected in comparison with biochar in as obtained form without a vast increase in production cost. Moreover, synthesis and characterization of biochar microparticles for adsorption is a relatively untouched area and further research is needed in this field. In general, nanoparticles agglomerate in liquid and which will reduce the adsorption capacity since agglomeration will reduce the effective surface area of the nanoparticles (Kalia et al., 2011). In the case of biochar microparticles, the

possibility for agglomeration can be ruled out in comparison with nanoparticles. In addition, because of its unique environmental properties, carbon nanoparticles are toxic to many organisms (Brar et al., 2010; Firme Iii and Bandaru, 2010). Hence, disposal of nanoparticles after utilization can be a major issue. While considering biochar as a waste management option and further producing “toxic nanoparticles”, it is not an effective method for waste reduction. Hence, biochar microparticles can be used as an effective non-toxic adsorbent as well as a waste management option.

In this study, the focus was to evaluate the general adsorption potential of biochar microparticles (derived from various sources) based on methylene blue (MB) adsorption experiments. MB was considered as a model for visible pollution as a result of its strong adsorption onto solids and toxicity to humans and animals (Sun et al., 2013). The equilibrium data of the adsorption process were used to study the adsorption mechanism of the MB molecules. This study also aimed to check the adsorption behavior of biochar microparticles.

2. Materials and Methods

2.1 Materials

Case studies of three biochar samples are presented. The first biochar sample (BC- PW) was obtained from Pyrovac Inc. (Quebec (Qc), Canada). BC-PW was derived from pine white wood (80% v/v) purchased from Belle-Ripe in Princeville and the rest was spruce and fir (20%). BC-PW was produced at 525°C under atmospheric pressure for 2 minutes in the presence of nitrogen and used as obtained from the reactor outlet. The second biochar sample (BC-PM) was obtained from Research and Development Institute for Agri-Environment”(IRDA), Quebec (Qc), Canada. This biochar was derived from solid fraction of pig slurry and prepared at 400 °C for 2 h at 15°C/min in the presence of nitrogen at a flow rate of 2 L min^{-1} during heating. The third biochar sample (BC – PD) was prepared at INRS – ETE (Quebec (Qc), Canada) and it was obtained from cardboard waste material via pyrolysis technique. Pyrolysis was performed at 500°C at 15°C/minute in the presence of nitrogen at a flow rate of 2 L min^{-1} and for 2 hours. Pine wood, pig manure and cardboard

materials commonly produced waste materials in Canada and which can be used for the production of biochar without any further pre-treatment. Powdered activated carbon (AC) was used as positive control for the adsorption studies to compare the adsorption results and was purchased from Fisher scientific (Ottawa, Canada).

Methylene blue dye (MB) shows strong adsorption to solids and it is widely recognized for its usefulness in characterizing materials (Cheng et al., 2013; Inyang et al., 2014) and the dye was purchased from Fisher scientific (Ottawa, Canada). A stock solution of 1000 mg L^{-1} MB was prepared in an amber colored volumetric flask and diluted to the required concentrations (500–10 mg L^{-1}) in deionized water. MB used in this study was analytical grade

2.2 Biochar microparticles preparation

Biochar microparticles were prepared from all the aforementioned biochar samples with a series of size reduction using mortar and pestle. Subsequently, the particles were sequentially sieved through ASTM 20, 50 and 200 numbered sieves for 10 minutes and particle size obtained was 850-300µm (S1- large sized), 300-75µm (S2-medium sized) and less than 75µm (S3- microparticles). In comparison with other methods, such as vapour deposition method for nanoparticle production, dry grinding method does have its own advantage, such as cost-effectiveness and simplicity.

2.3 Biochar and microparticles characterization

Particle size distribution analysis of microparticles was performed using a Horiba particle size analyzer (LA-950 Laser Particle Size Analyzer, HORIBA, Edison, NJ, USA). The analysis was carried out in triplicate and the mean value was taken. The specific surface area of each sample was also obtained from microparticle size analyzer and was expressed in cm^2/cm^3 of water. The surface characteristics, such as porosity and pore distribution were analyzed using field emission-scanning electron microscopy. Particles were coated with gold (plasma state) prior to the analysis to minimize

sample charging. The ash and moisture content of BC-PW, BC-PM, and BC-PD was analyzed as per ASTM methods (ASTM D1762 – 84).

2.4 Adsorption studies

Adsorption experiments were performed to determine and compare the adsorption potential of different biochar samples. Prior to analysis, the samples were dried overnight at 60 ±1°C in an oven and then cooled in a desiccator to remove any moisture present and the final moisture content was assumed to be negligible.

Initially, the effect of biochar dose of each biochar (BC-PW, BC-PM, and BC-PD) on the adsorption process was investigated by using different biochar concentrations from 3-15 g L^{-1} at 50 mg L^{-1} of MB concentration. The adsorption studies were carried out for 120 minutes in an incubator shaker at 25±1 $^{\circ}$C and 150 rpm.

The effect of varying concentrations of MB on the adsorption process was studied using all the three biochars and their corresponding size reduced particles and microparticles. Various concentrations used for the study were 10 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1} and 500 mg L^{-1} and the experiments were carried out in triplicate. In this study, other parameters, such as pH (6.5) and agitation speed were kept constant (150 rpm) and 0.3 g of biochar was added to Erlenmeyer flasks containing 30 mL of MB and the flasks were placed in incubator shaker for 120 minutes.

After 120 minutes, the suspensions were centrifuged at 37 000 *x* g for 15 minutes to get a clear liquid and the supernatant was collected by decantation. The remaining concentration of MB in the solution was measured at 665 nm using a UV–visible spectrophotometer (Epoch™-Biotek). The amount of adsorbed MB at time t (mg g^{-1}) was calculated using Equation 1

$$q_t = V(C_0 - C_t)/w \quad (1)$$

Where, C_0 and C_t (mg L^{-1}) are the liquid-phase initial and final concentrations of MB respectively. V (L) is the volume of the MB solution, and w (g) is the mass of dry biochar used.

2.4.1 Adsorption isotherm models

Adsorption studies were carried out using 0.3 g of the raw biochar samples and activated carbon with concentrations ranging from 10 to 500 mg L^{-1} at a constant temperature of 25 °C in an incubator shaker at 150 rpm for 24 hours to get an equilibrium concentration. The obtained data were plotted using Langmuir and Freundlich adsorption isotherm models. Linearized forms of the equations were applied for the adsorption isotherms.

Langmuir isotherm model was constructed based on the assumption of homogeneous monolayer adsorption onto the surface with no re-adsorption of adsorbate on the surface and can be written as Equation 2 (Tan et al., 2009; Weber and Chakravorti, 1974).

$$C_e/q_e = 1/Q_0K_L + C_e/Q_0 \quad (2)$$

Where C_e (mg L^{-1}) is the equilibrium concentration of the adsorbate, q_e (mg g^{-1}) is the amount of adsorbate adsorbed per unit mass of adsorbent, Q_o and K_L are Langmuir constants related to adsorption capacity and rate of adsorption, respectively. When C_e/q_e is plotted against Ce, a straight line with slope of $1/Q_0$ and intercept of $1/Q_0K_L$ is obtained.

Freundlich isotherm model assumes heterogeneous adsorption on the surface of the adsorbent. According to Freundlich isotherm model, the stronger binding sites on the surface are occupied first and that the binding strength decreases with the increasing degree of site occupancy and which reduces the adsorption with time. The logarithmic form of the equation can be written as (Freundlich, 1906; Tan et al., 2009)

$$\log q_e = \log K_F + {}^{1}/_{n} \log C_e \quad (3)$$

The plot of log q_e versus log C_e gives a straight line with slope of 1/n and intercept of log K_F. Where K_F is the adsorption capacity of the adsorbent and n is the favourability factor of the adsorption. The adsorption process becomes more heterogeneous when the slope 1/n equals zero.

3. Results and Discussion

3.1 Biochar characterization

3.1.1 Moisture, ash content and chemical composition

The moisture and ash content of each biochar is given in Table 1. Biochar molecules show hydrophobic character. On the contrary, at least in few cases biochar molecules are hygroscopic in nature and most of the biochars exhibit significant adsorption capacity for water vapor (Ahmad et al., 2012). The hygroscopic nature of biochar may be due to water of hydration within the ash present in the biochar or it may also be due to water molecules associated with the organic portions of the biochar, including adsorbed water vapor (Inyang and Dickenson, 2015). The amount of moisture in biochar may vary according to its method of production and storage (Ahmad et al., 2012). In this study, BC-PM (7.65%) exhibited maximum moisture content of the samples followed by BC-PD and BC-PW (4.9 % and 1.5% respectively).

Biochar prepared from livestock manure, such as pig manure usually contains high ash content (Cao and Harris, 2010; Cao et al., 2011) and in this study, pig manure biochar exhibited high amount of ash content at 65.48 %. Plant derived biochar usually contains low amounts of ash (Ahmad et al., 2014; Boateng et al., 2010). In this study, BC-PD and BC-PW originating from plant sources exhibited lower amounts of ash contents, such as 12.45 % and 9.52 %, respectively. The impact of ash content on adsorption was seldom studied and exact effects were not clearly understood for each contaminant. It is reported that biochar with high ash content exhibited subdued adsorption

potential for organic pesticides, such as carbaryl and atrazine because adsorption sites of organic moieties can be masked by ash (Inyang and Dickenson, 2015; Zhang et al., 2013).

Generally, the pine wood feedstock contains cellulose (about 40.7 %) hemicellulose (about 26.9%) and lignin (about 27 %) (Wang et al., 2015). In this study, for preparing biochar sample BC-PW, the feedstock used was scots red pine and typical composition included the previously mentioned components. The chemical composition of the feedstock used for the preparation of pig manure biochar (BC-PM) has been given in a previous publication (Fernandez-Lopez et al., 2015). The chemical composition data of paper derived biochar (BC-PD) used for this study has also been given in a previous publication (Ghorbel et al., 2015)

3.1.2 SEM analysis

SEM images of BC-PW, BC-PM and BC-PD and activated carbon are shown in Fig 1. Moreover, SEM images of each subclass of biochar microparticle (S1, S2, S3 for BC-PW, BC-PM and BC-PD) is given as supplementary data. A representative microparticle or a group of microparticles are used for the SEM images. BC-PW displayed well-arranged pores on the surface in rows (Fig 1.A). On an average, from the SEM visual data, the pore size appeared to be 5 µm (Fig 1.B). For BC-PM, the pores were irregularly arranged (Fig 1.C) and the pore size varied between 4 and 5 µm (Fig 1.D).The sample exhibited an uneven and rough surface texture. BC-PD exhibited a fibrous structure which lacked the presence of proper pores (Fig 1.E) and these irregular cracked and short fibers mixed with aggregates were typical for biochar derived from cellulose (Méndez et al., 2009). However, due to the fibrous structure make-up, the total surface area will be very large. The criss-cross arrangement of biochar fibers increases the total surface which in turn resulted in better surface area as shown in Table 4. Even though BC-PD lacked the presence of well-defined pores, some pits and pore like structures were visible on the surface due to criss-cross arrangement of fibers. All the samples exhibited potential for the adsorbate molecules to be trapped and adsorbed by the biochars with higher surface area and presence of pores.

3.1.3 Microparticles and particle size distribution

The biochar microparticles were prepared by size reduction and sequentially sieving through ASTM sieves. The results obtained for particle size and size distribution are given in Table 2. Fig.5.A presents particle size distribution of as obtained biochar and Fig.5.B presents the particle size distribution of the microparticles (S3) prepared from each class of biochar sample.

For BC-PW sample, the microparticles (S3) were well within the size distribution defined by IUPAC. The mean size of the particles was 55.66 ± 47.71. About 90% of the microparticles were lower than 118.08 µm and 10% of the particles were lower than 15.96 µm. The geometric mean of the particle size was found to be 41.26 µm with a median size of 38.93 µm. Hence, the prepared particles were confirmed to be microparticles. S1 and S2 samples were also well in the range of production with mean sizes of 635.16±342.32 µm and 250.34±141.43 µm, respectively.

Microparticles (S3) prepared from BC-PM showed a mean size of 56.49 ±38.49 µm. About 90% of the microparticles were lower than 105.49 µm and 10% of the particles were lower than 17.18 µm in size. The geometric mean size was found to be 45.10 µm with a median size of 48.31 µm. S1 and S2 class exhibited a mean size of 415.14 ±308.64 µm and 212.51± 141.27 µm, respectively.

Microparticles (S3) prepared from BC-PD were analyzed with a mean size of 61.33 ± 47.03 µm. About 90% of the microparticles were lower than 121.33 µm and 10% of the particles were lower than 20.61µm. The geometric mean size was found to be 48.45 µm with a median size of 46.84 µm. The mode size of the particles was calculated to be 48.02 µm. S1 and S2 samples were also well in the range of production with mean sizes of 382.53 ± 298.98 µm and 205.62± 138.41 µm, respectively.

3.2 Adsorption studies

3.2.1 Effect of adsorbent dose

MB removal efficiencies with an initial MB concentration of 50 mg L^{-1} at 25 °C by BC-PW, BC-PM and BC-PD are shown in Fig.2. The removal efficiency of MB increased with biochar dose at a constant MB concentration. BC-PW exhibited almost 9 fold (885 %) increase in adsorption with a 5 folds increment in adsorbent dosage. For BC-PM and BC–PD, the effect of increase in adsorbent dosage was clearly demonstrated, an increase in percent of adsorption being 14 times and 22 times for BC-PM and BC–PD, respectively for the same increase in dosage. The increase in removal efficiency can be attributed to the increase in available sorption surface (Ahmad et al., 2014). A similar trend of increasing adsorption capacity with adsorbent dosage was shown in previous studies with biochar prepared from palm bark and eucalyptus (Inyang et al., 2014; Sun et al., 2013). Due to the fibrous surface (Fig 1.E) and hence increased surface area, BC-PD displayed good abilities for adsorption with increased adsorbent dosage. As the particle size was kept constant, the surface area was directly proportional to the mass of adsorbent in the solution and hence the adsorption was increased.

3.2.2 Adsorption isotherms - data analysis

The adsorption data were analyzed using Langmuir and Freundlich adsorption isotherm models and the data is given in Fig. 3. The isotherm parameters calculated from the models are given in Table 3.

Langmuir isotherm exhibited good agreement for BC-PM and BC-PD with R^2 values 0.989 and 0.995, respectively. For the same adsorbents, the correlation with Freundlich isotherm model was lower and the R^2 values were 0.811 and 0.070 for BC-PM and BC-PD, respectively. This perfect fit of the adsorption data with Langmuir isotherm model for BC-PM and BC-PD indicated that the adsorption was monolayer which involved chemical and physical adsorption. From previously reported studies (Kołodyńska et al., 2012; Mohan et al., 2014), biochar prepared from pig manure exhibited excellent ability to remove metals from water and the mechanism was explained by

Langmuir adsorption models. This study also pointed towards the same mechanism for methylene blue adsorption biochar prepared from the pig manure (BC-PM). To the best of our knowledge, adsorption mechanism data was not available for the biochar derived from paper and related materials and for the first time this study reported that the adsorption will be monolayer for methylene blue.

For BC- PW and commercially obtained powdered activated carbon, the experimental data presented good agreement with Freundlich isotherm models at R^2 values of 0.997 and 0.955, respectively. Meanwhile, the value of the exponent n was greater than 1 for both of the adsorbents confirming the favorable adsorption conditions. These values pointed towards the possibility of heterogeneous adsorption with physical forces of binding. The results obtained for pine wood derived biochar (BC- PW) displayed good agreement with the mechanisms previously studied (Cheng et al., 2013; Mohan et al., 2007) with possible heterogeneous adsorption.

3.2.3 Microparticles and adsorption

Biochar BC-PW

The adsorption data with varying MB concentrations for the microparticles is given in Fig 4.A .The surface area of the adsorbents is given in Table 4 .The adsorption capacity (mg g^{-1}) was increased when the MB concentration increased from 10 to 500 mg L^{-1}. This primarily showed physical adsorption process. The size reduction of biochar increased adsorption capacity. The effective surface area gradually increased with the size reduction and hence the available sites for adsorption also increased. This trend can be seen with concentrations from 10 to 500 mg L^{-1}.

Also, the adsorption nearly increased 100 % in all cases when compared with the obtained biochar and microparticle (S3). Hence, the microparticles clearly improved the surface area which in turn increased the adsorption. A maximum of 16.75 mg g^{-1} adsorption capacity was observed in previous studies for pine derived biochar (Cheng et al., 2013), whereas in this study, the maximum adsorption capacity observed was 7.9±0.5 mg g^{-1} and activated carbon exhibited excellent adsorption with

48.3±3.6 mg g^{-1} at 500 mg L^{-1}initial concentration. This decrease can be due to the difference in original materials and the preparation conditions. In fact, it was reported that feedstock and pyrolysis conditions can influence molecular structure and pores size distribution of biochar, which consequently affects biochar sorption characteristics (Ahmad et al., 2012; Cantrell et al., 2012; Gai et al., 2014). Despite these limitations, significant enhancement in adsorption occurred with prepared microparticles.

Biochar BC-PM

The data for BC-PM microparticles and varying MB concentrations is given in Fig. 4B and the surface area of the adsorbents is provided in Table 4. As compared to BC- PW, this biochar appeared to be more effective for MB adsorption. BC-PM exhibited similar trend in adsorption as biochar BC-PW. An increasing trend was observed with increasing surface area along with microparticles. Also the adsorption capacity (mg g^{-1}) was further increased with an increase in adsorbate concentration (Fig. 4.B). Biochar with high ash content usually exhibits reduced adsorption potential for organic contaminants. On the contrary, in this study, pig manure biochar (BC-PM) with relatively higher ash content exhibited higher adsorption which can be attributed to the cationic properties of methylene blue and the general anionic properties of biochar derived from pig manure. The physico-chemical adsorption process via Langmuir model also points towards the same possibility. The increase in concentration increased adsorption as the number of adsorbate molecules were increased which produced enhanced interactions between the adsorbent and adsorbate and hence the adsorption was increased. The increase in total surface area increased the adsorption capacity of the size reduced particles (Inyang and Dickenson, 2015) .

On an average, microparticles (S3) showed 35% increase in adsorption efficiency (mg g^{-1}) despite the initial concentration. When compared to BC-PW, the microparticles were not effective for an enhanced adsorption. However, at a concentration ranging from 10 to 100 mg L^{-1}, BC-PM microparticles exhibited almost same adsorption capacity as that of activated carbon. This indicated

that at lower initial concentrations, size reduction can be an effective process for adsorption with pig manure derived biochar, and which can replace the expensive activation process (Kołodyńska et al., 2012; Kambo and Dutta, 2015; Inyang et al., 2014).

Biochar BC-PD

The adsorption data for this category of biochar is given in Fig. 4.C and the surface area of the adsorbents is entailed in Table 4. A similar trend in adsorption was observed for BC-PD as biochar BC-PW and biochar BC-PM. The maximum adsorption capacity obtained was 8.9 ±0.9 mg g^{-1}.

Biochar BC-PD appeared to be very effective at lower concentrations and the microparticles (S3) produced almost same results as the commercially available activated carbon at 10 mg L^{-1} concentration. Irrespective of the particle size at lower concentrations (10 mg L^{-1} and 50 mg L^{-1}), all of them were very effective for MB adsorption so that equilibrium was attained within 120 minutes at lower concentrations, such as 10 mg L^{-1}. Also, at higher concentrations, such as 500 mg L^{-1}, the increase in adsorption capacity of microparticles from raw biochar was very high as 800 %, but the total adsorption capacity (mg g^{-1}) was very less when compared to the commercially available activated carbon. The increase in total surface area (microparticle) and number of adsorbate molecules (elevated concentration) produced increased interactions between the adsorbent and adsorbate and hence the adsorption was increased (Inyang and Dickenson, 2015).

In general, all biochar samples exhibited potential for adsorption. In addition, microparticles were highly effective at lower concentrations, such as 10 mg L^{-1} and 50 mg L^{-1} when compared to commercially available activated carbon. In this study, BC-PM and BC-PD microparticles exhibited excellent abilities to adsorb organic contaminant (MB) from water at lower concentrations and which is an important property since under actual problematic conditions, the concentrations of some organic contaminants may be lower and toxicity at lower concentration was reported to be very high for emerging contaminants. The mechanism of adsorption also varied with the source material for the preparation of biochar and the method of production. From isotherm models, BC-PM and BC-PD

exhibited monolayer adsorption possibilities followed by perfect fit with Langmuir isotherm model whereas BC-PW and activated carbon displayed heterogeneous adsorption corroborated by Freundlich isotherm model.

4. Conclusions

Biochar microparticles can be prepared from biochar using economically feasible method. In this study, the microparticles prepared from three different types of biochar exhibited enhanced adsorption capacities (0.975 mg g^{-1}) at reduced concentrations of 10 mg L^{-1}, which were comparable with the commercially available activated carbon (0.980 mg g^{-1}) used for the study. The adsorption capacities of biochar varied with the source material and method of preparation. The adsorption capacity for cationic dye methylene blue also increased with the increase in surface area of the microparticles. Maximum adsorption capacity (Q_{max}) was observed to be 3.99 mg g^{-1}, 16.30 mg g^{-1} and 1.66 mg g^{-1}, respectively for pine wood, pig manure and paper derived biochar microparticles at MB concentration of 50 mg L^{-1}. The Langmuir and Freundlich adsorption isotherm models were used to denote the adsorption of the methylene blue. The equilibrium data were well described by Langmuir model for BC-PM and BC-PD and Freundlich model for BC-PW.

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Table 1: Ash content and moisture content

Biochar	Moisture (%)	Ash content (%)
Pine wood biochar (BC-PW)	1.53±0.1	9.52±0.8
Pig manure biochar (BC-PM)	7.65±0.4	65.48±1.8
Paper derived biochar (BC-PD)	4.99±0.3	12.45±1.1

Table 2: Micro particles- Particle size distribution

Biochar		Mean size (µm)	D10 (µm)	D90 (µm)
Pine wood biochar (BC-PW)	850-300 µm (S1)	635.16±342.32	277.29	1073.78
	300-75 µm (S2)	250.34±141.43	93.87	438.03
	>75 µm (S3)	55.66±47.71	15.96	118.08
Pig manure biochar (BC-PM)	850-300 µm (S1)	415.14±308.64	76.28	833.27
	300-75 µm (S2)	212.51± 141.27	64.69	398.89
	>75 µm (S3)	56.49±38.49	17.18	105.49
Paper derived biochar (BC-PD)	850-300 µm (S1)	382.53± 298.98	59.97	785.75
	300-75 µm (S2)	205.62±138.41	62.48	388.83
	>75 µm (S3)	61.33±47.03	20.61	121.33

Table 3: Langmuir and Freundlich adsorption isotherm constants

Biochar Sample	Langmuir constants			Freundlich constants		
	Q_{max} (mg/g)	K_L (L/mg)	R^2	K_F (mg/g)	n	R^2
Pine wood	3.99	0.015	0.880	1.540	1.22	0.997
Pig manure	16.30	0.526	0.989	0.044	1.98	0.811
Paper derived	1.66	0.134	0.995	0.219	16.50	0.070
Activated carbon	48.30	3.790	0.223	0.586	1.29	0.955

Table 4: Specific surface area of various biochars

Sample	Specific surface area obtained from particle size analyzer (cm^2/cm^3) in water
BC-PW	198.03±11.5
BC-PW S1	263.43±20.4
BC-PW S2	373.89±30.1
BC-PW S3	1894.2±55.6
BC-PM	346.97±18.4
BC-PM S1	384.45±31.3
BC-PM S2	569.60±48.3
BC-PM S3	1696.2±88.7
BC-PD	310.82±21.0
BC-PD S1	424.92±36.7
BC-PD S2	511.30±46.2
BC-PD S3	1530.8±88.3
AC	3130.3±67.6

BC-PW: Pine wood biochar; BC-PM: Pig manure biochar; BC-PD: Paper derived biochar; AC: Activated carbon (microparticles S1: (850-300) μm; S2: (300-75) μm; S3: >75 μm)

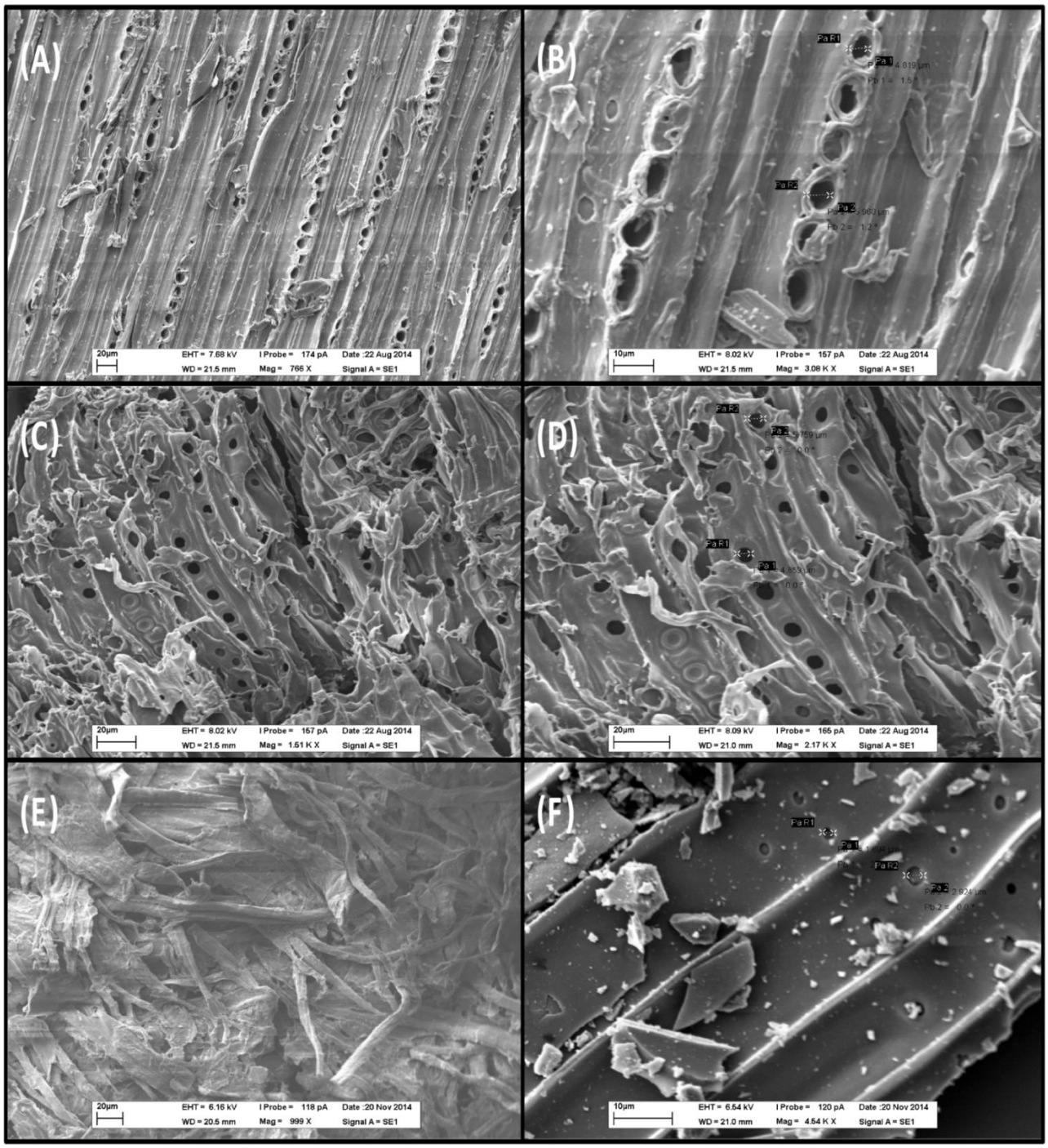

Figure 1: Scanning electron microscopy images of biochar: (A) Pine wood biochar (BC-PW); (B) BC-PW porosity; (C) Pig manure biochar (BC-PM); (D) BC-PM porosity; (E) Paper derived biochar (BC-PD); (F) activated carbon

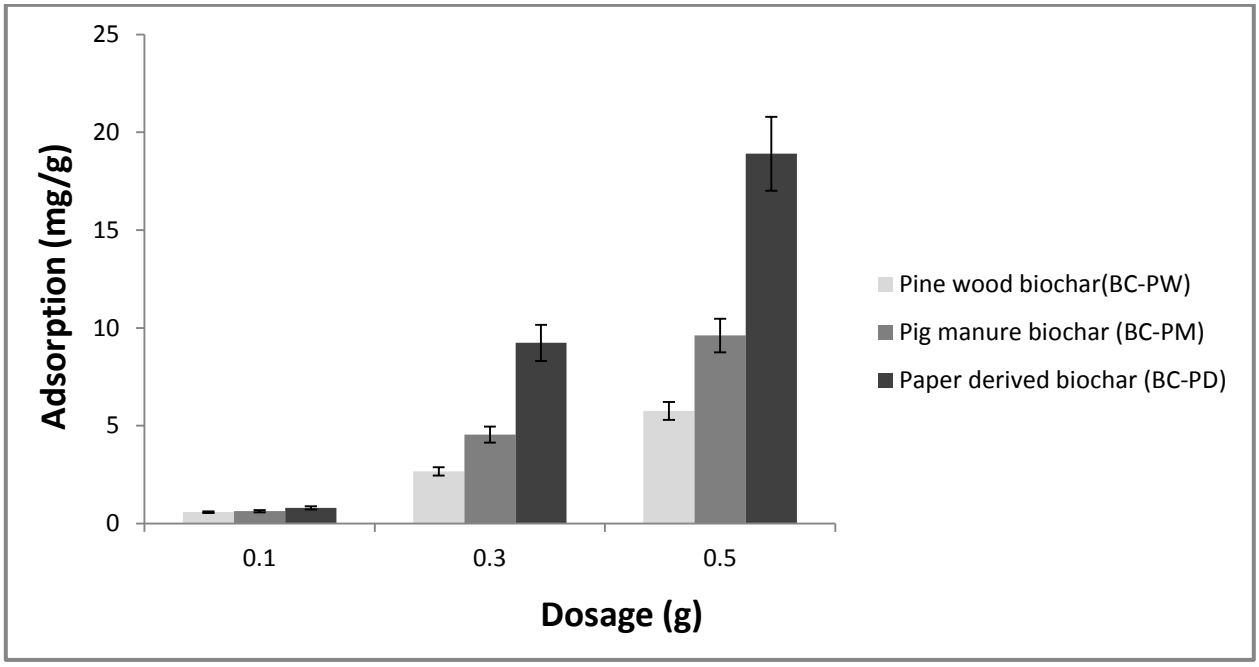

Figure 2: Effect of adsorbent dosage on adsorption capacity

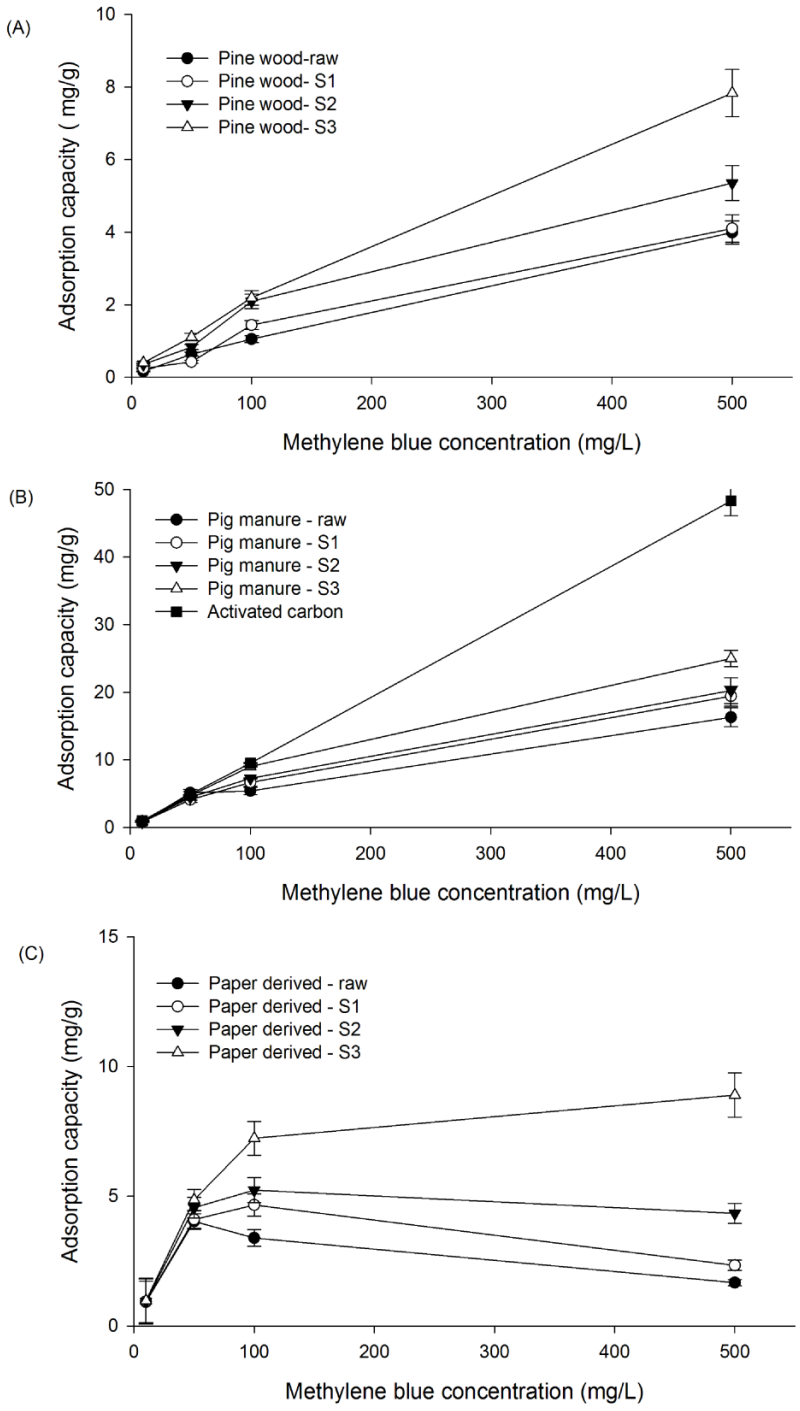

Figure 3 : Adsorption capacity of Biochar microparticles: (A) Pine wood biochar (BC-PW); (B) Pig manure biochar (BC-PM); (C) Paper derived biochar (BC-PD); microparticles S1: (850-300) µm; S2: (300-75) µm; S3: >75 µm

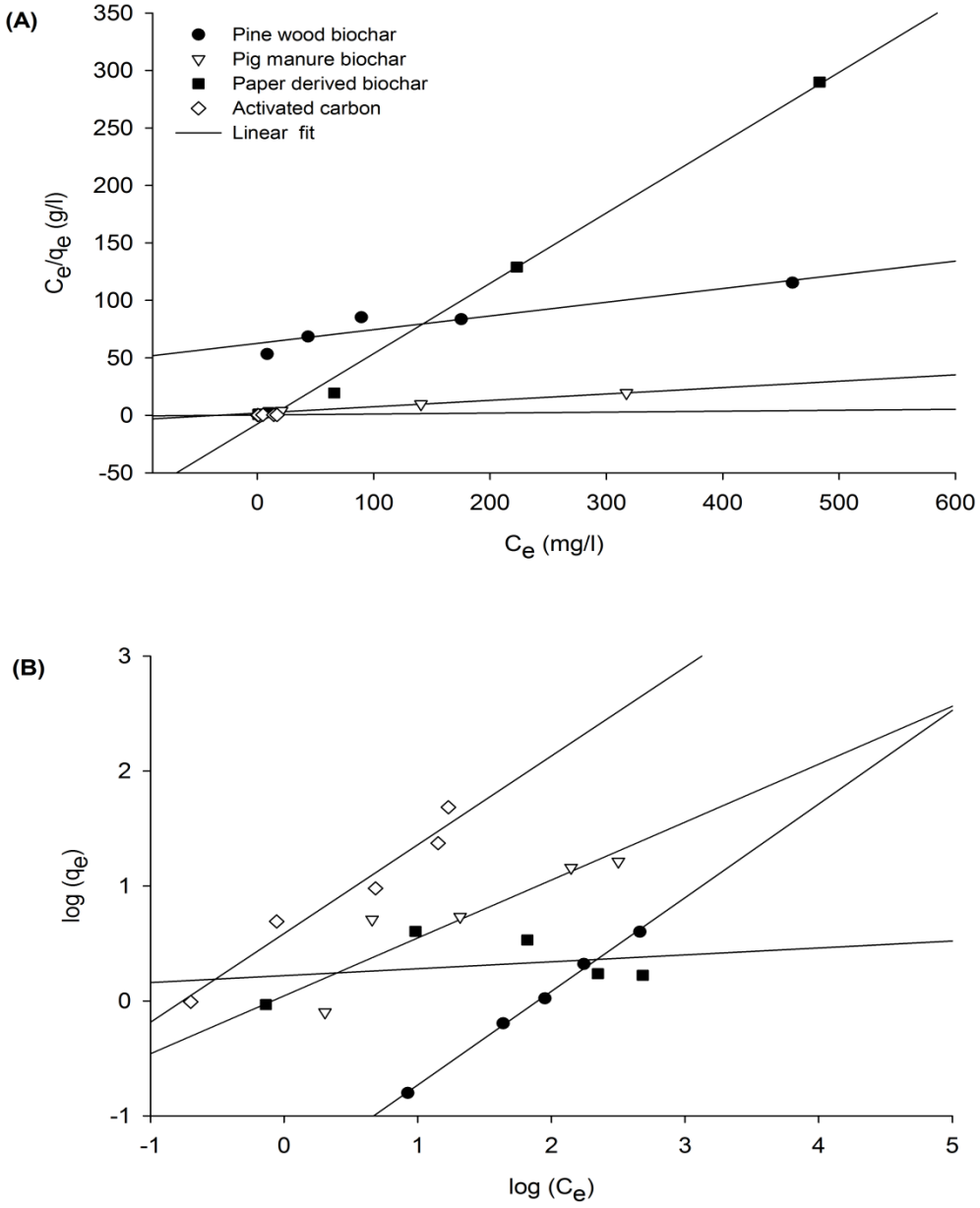

Figure 4: Adsorption isotherms of different biochar samples: (A) Langmuir adsorption isotherm; (B) Freundlich adsorption isotherm (C_e : equilibrium concentration of the adsorbate (mg L^{-1}), q_e : amount of adsorbate adsorbed per unit mass of adsorbent (mg g^{-1}))

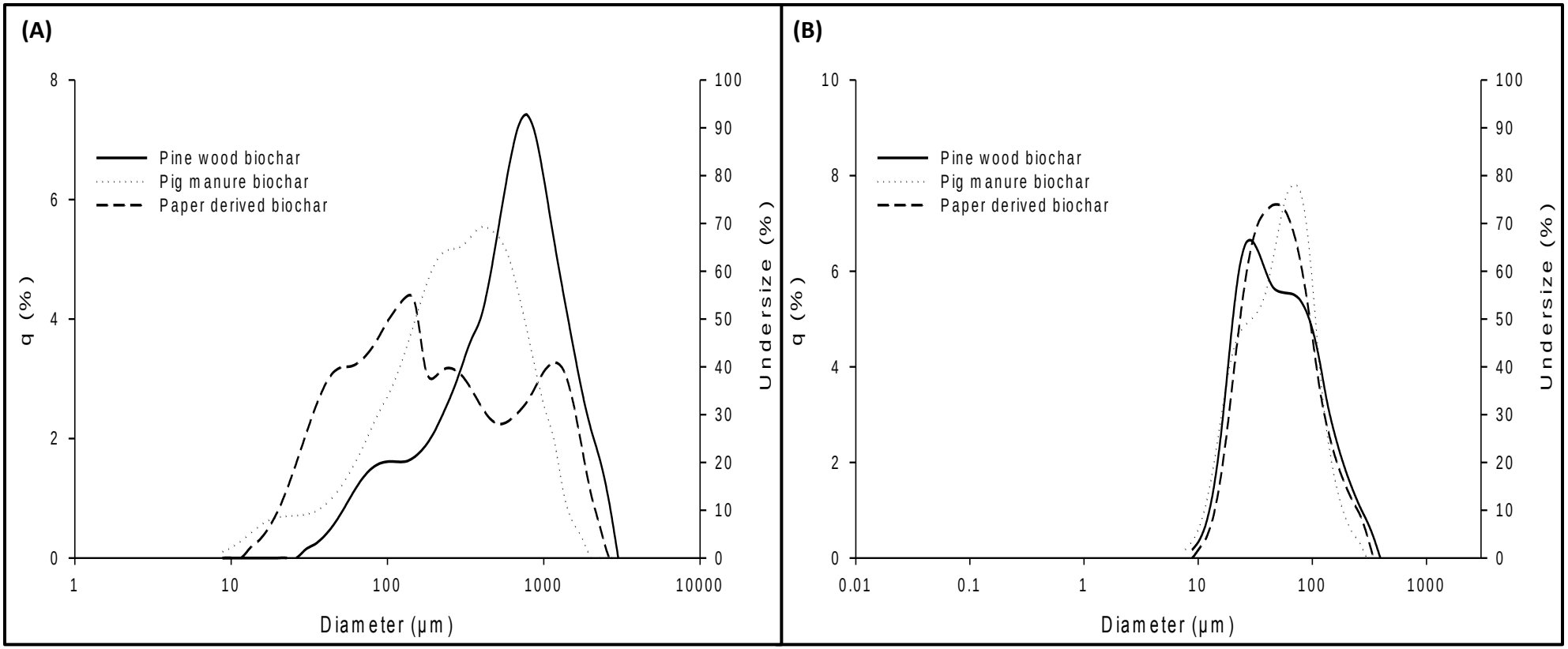

Figure 5: Particle size distribution of biochar: (A) as obtained; (B) biochar microparticles

PART 2

AN INSIGHT INTO THE ADSORPTION OF DICLOFENAC ON DIFFERENT BIOCHARS: MECHANISMS, SURFACE CHEMISTRY, AND THERMODYNAMICS

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RÉSUMÉ

Les biochars ont été préparés à partir de bois de pin et de fumier de cochons. Les microparticules de biochar obtenues par broyage ont été évaluées pour l'enlèvement du contaminant émergent diclofenac (DCF) et les mécanismes sous-jacents ont été étudiés en profondeur. La caractérisation du biochar s'est effectué avec l'utilisation d'un analyseur de particules, des équipements SEM, BET, FT-IR, XRD et XPS, et d'un potentiomètre. Le biochar de fumier de cochon (BC-PM) a manifesté d'excellente capacité d'enlèvement (99.6%) en comparaison avec le biochar de bois de pin (BC-PW) à des concentrations de 500 µg L-1 of DCF (concentration significative dans l'environnement). La diffusion intraparticule s'est avérée être le processus de facilitation majeur de l'adsorption. Le BC-PW a suivi une cinétique d'adsorption de premier ordre alors que le BC-PM a plutôt suivi une cinétique de pseudo second ordre. Le biochar de bois de pin a été significativement affecté par les variations de pH alors que pour le biochar de fumier de cochon, l'effet du pH était minime due à ses groupes fonctionnels en surface et l'hydrophobicité du DCF. La thermodynamic, la présence d'ions co-existant, la concentration initial de DCF and la taille des particules ont joué un rôle significatif dans l'adsorption du DCF. Plusieurs modèles isothermes ont aussi été étudiés et les résultats sont présentés dans l'étude.

Mots clés : Diclofenac; adsorption; biochar; chimie d'interface; thermodynamique; mécanisme

ABSTRACT

Biochars were prepared from feedstocks pinewood and pig manure. Biochar microparticles obtained through grinding were evaluated for the removal of emerging contaminant diclofenac (DCF) and the underlying mechanism were thoroughly studied. Characterization of biochar was carried out using particle size analyzer, SEM, BET, FT-IR, XRD, XPS and zeta potential instrument. Pig manure biochar (BC-PM) exhibited excellent removal efficiency (99.6%) over pine wood biochar (BC-PW) at 500 µg L-1 of DCF (environmentally significant concentration). Intraparticle diffusion was found to be the rate limiting process. BC-PW followed pseudo first-order kinetics whereas BC-PM followed pseudo second –order kinetics. Pine wood biochar was largely affected by pH variations whereas for pig manure biochar, pH effects were minimal owing to its surface functional groups and DCF hydrophobicity. Temperature, presence of co-existing ions, initial adsorbate concentration and particles size played substantial role in adsorption. Various isotherms models were also studied and results are presented.

Keywords: Diclofenac; Adsorption; Biochar; Interface chemistry; Thermodynamics; Mechanisms

1. Introduction

Emerging contaminants (ECs) are gaining wider attention nowadays due to their potential environmental impacts. Even though ECs are detected at fairly low concentrations, the adverse effects of ECs on several organisms are high and usually uncertain for several ECs. Among the emerging contaminants, pharmaceutical, diclofenac (DCF) is often detected in wastewater treatment plant influent, effluent, surface water and drinking water (Farré et al., 2008; Heberer, 2002; Stuart et al., 2012). It is estimated that about 1443 ± 58 tons of DCF are consumed globally on an annual basis (Acuña et al., 2015), which makes it as the 12th bestselling generic molecule globally worth $1.61± 15% billion sales per year (Palmer, 2012). This large consumption, in turn, resulted in the ubiquitous presence of DCF in wastewater. The incomplete degradation of DCF in wastewater treatment plants leads to its presence in surface water and even in drinking water (Vieno & Sillanpää, 2014). This presence in water resources causes toxic concerns towards several aquatic and terrestrial organisms (Lonappan et al., 2016a). It has been reported that even at 250 ng L^{-1}, DCF can induce tissue damages in several mussel species and at 1 µg L^{-1}, cytological alterations in rainbow trout (Ericson et al., 2010). Thus, even very small concentrations of DCF can lead to a considerable effect on the aquatic ecosystems.

In the recent decades, adsorption has emerged as an effective treatment method for the removal of various organic emerging contaminants, such as pharmaceuticals (Nam et al., 2014; Westerhoff et al., 2005). Activated carbons from the various origin are the conventional adsorbents studied for the removal of ECs (Westerhoff et al., 2005). The physicochemical properties of adsorbents and adsorbates, such as, hydrophobicity, surface functional groups, pore size, external surface area and chemical composition determine the removal efficiency along with other experimental parameters, such as pH and temperature (Lonappan et al., 2016c; Nam et al., 2014; Nielsen et al., 2014). However, all adsorbents even after surface activation cannot become excellent adsorbents for all contaminants. For instance, most of the activated carbons will exclude larger molecules (>3000 Da) due to its small pore size (<2 nm) (Kilduff et al., 1996). Moreover, the activation process is an

additional procedure and incurs a cost. Thus, economical and eco-friendly adsorbents must be specifically identified for the compounds of interest to be treated. As a waste management alternative, carbonaceous material biochar exhibited its potential towards sustainability and demonstrated its usefulness as a value-added product for several environmental applications (Glaser et al., 2009; Lonappan et al., 2016c; Woolf et al., 2010). Biochar is carbon negative (Glaser et al., 2009) material and hence the production and consumption are appealing towards a sustainable future. For biochar, the adsorption mechanisms and adsorption capacity significantly vary with feedstock and method of production as a result of changes in the chemical properties and surface chemistry. Pinewood and pig manure are two of the largest produced agricultural and farming industry residues in Quebec, Canada; which needed proper recycling/disposal. Hence, production of biochar from these materials is an excellent waste management and valorization alternative. Moreover, biochars of this category are well known for its richness in micropores and adsorption abilities (Lonappan et al., 2016c). The porous structure and surface chemistry features make biochar an excellent adsorbent for several environmental contaminants. Due to the aromaticity and hydrophobicity, biochar is an excellent sorbent for a number of hydrophilic organic compound and inorganic compounds(Fang et al., 2014). A proper understanding of the interactions between adsorbate and adsorbent is necessary to design efficient adsorption systems for the removal of contaminants.

The aim of this study is to evaluate the adsorption efficiency and adsorption processes of two carbonaceous materials; biochar derived from pinewood and pig manure for the removal of a model emerging contaminant, DCF for the first time. Biochar microparticles were prepared and characterized by particle size analyzer, scanning electron microscope, Fourier transform infrared spectroscopy, X-ray diffraction and Brunauer, Emmett and Teller (BET) analysis and zeta potential instrument. The underlying sorption processes, such as adsorption rate, adsorption model, and adsorbent/adsorbate interaction were studied using equilibrium adsorption data by various kinetic and isotherm models. The effect of adsorbent dosage, particle size and thermodynamic behavior of

adsorption were also studied. Moreover, the effect of surface charges on adsorption was studied by pH variations and zeta potential measurements. The result presented explains the effect of feedstock, chemical and surface features of biochar for the effective removal of DCF.

2. Material and methods

2.1. Materials

Pinewood biochar sample (BC- PW) was obtained from Pyrovac Inc. (Quebec, Canada). BC-PW was derived from pine white wood (80% v/v) purchased from Belle-Ripe in Princeville and the rest was spruce and fir (20%). BC-PW was produced at 525±1°C under atmospheric pressure for 2 minutes in the presence of nitrogen (to create oxygen free environment) and used as obtained from the reactor outlet. The second biochar sample prepared from pig manure (BC-PM) was obtained from “Research and Development Institute for Agri-Environment” (IRDA), Quebec, Canada. BC-PM was derived from the solid fraction of pig slurry and prepared at 400±1 °C for 2 h at 15°C min^{-1} in the presence of nitrogen at a flow rate of 2 L min^{-1} during heating. Powdered activated carbon (AC) was used as positive control for the adsorption studies to compare the adsorption results and was purchased from Fisher scientific (Ottawa, Canada). At a time about 200 grams of raw biochar samples was used for microparticle production. Samples were ground using ‘Retsch RS 200’ vibratory disc mill at 750 rpm for 2 minutes for BC-PW and 1 minute for BC-PM. Subsequently, the obtained particles were sieved through ASTM 200 numbered sieve (corresponds to less than 75µm particle size) for 10 minutes. The particle size and size distribution were further confirmed using Horiba particle size analyzer (LA-950 Laser Particle Size Analyzer, Horiba, Japan).

Diclofenac sodium salt (98%; CAS 15307-79-6) was purchased from Fisher Scientific (Ottawa, ON, Canada). An internal standard (IS), diclofenac-d_4 was obtained from C/D/N isotopes Inc. (Montreal, QC, Canada). The molecular weight of diclofenac is 296.14864 g/mol. Other properties are: log K_{ow} = 4.51 and pK_a = 4.15.

All the experiments were carried out using Milli-Q water (18 MΩ cm^{-1} at 25 °C) and were prepared in the laboratory using Milli-Q/Milli-RO Millipore system (Milford, MA, USA). All the reagents used for analysis of DCF and for pH adjustment of samples were of analytical grade.

2.2 Methods

2.2.1 Physico-chemical properties of microparticles

Horiba particle size analyzer (LA-950 Laser Particle Size Analyzer, Horiba, Japan). was used to measure the particle size distribution of microparticles. The analysis was carried out in duplicate and the mean value was measured. Surface characteristics of prepared microparticles were investigated by EVO® 50 smart scanning electron microscope (SEM) (Zeiss, Germany). Specific surface area was obtained from Brunauer, Emmett and Teller (BET) N_2 adsorption isotherms at 77 K (Autosorb-1, Quantachrome, USA). The presence of functional groups on the surface of adsorbents was investigated by Fourier transform infrared spectroscopy (FT-IR; Perkin Elmer, Spectrum RXI, FT-IR instrument fitted with lithium tantalate ($LiTaO_3$) detector. Powder x-ray diffraction (XRD) patterns of the samples were recorded using Panalytical Empyrean XRD with monochromatized CuK alfa radiation (1.5418 A). The surface charge properties of adsorbents were investigated by zeta potential measurements, which were conducted at a different equilibrium pH using a Nano-ZS Zetasizer (Malvern Instruments Inc., UK). Samples were pre-equilibrated to different pH (2, 5, 7, 10 and 13) maintaining a constant biochar dosage of 2 g L^{-1}. The zeta potential was measured in duplicate at each pH and the mean was calculated.

2.2.2 Batch adsorption studies

Batch adsorption studies were carried out in 125 mL conical flasks with 50 ml of sample in an INFORS HT - multitron standard shaking incubator (INFORS, Mississauga, Canada). The agitation was carried out at 200 rpm and kept constant throughout the study. The temperature was set to be 25 ±1°C and 0.1g of biochar was used for the study at pH 6.5 (unless stated otherwise for a

particular study). Prior to analysis, the biochar samples were dried overnight at 60 ±1°C in an oven and then cooled in a desiccator to remove any moisture present. After adsorption, DCF samples were separated using centrifugation at 11600 *x* g for 10 minutes in a MiniSpin® plus centrifuge. Post centrifugation, the supernatant was analyzed for DCF left in the liquid.

The adsorption capacity at time t (in μg g^{-1}) was calculated using Equation 1:

$$q_t = V(C_0 - C_t)/w \quad \text{---------- (1)}$$

Percentage of DCF removal at time 't' was calculated using Equation 2

$$\%\, removal\ (\%R) = \left\{\left(\frac{C_0 - C_e}{C_0}\right) 100\right\} \text{---------- (2)}$$

Where C_0, C_t and C_e represent the concentrations of DCF (mg L^{-1}) at the beginning of the experiment, at time t (min), and at equilibrium respectively; V is the volume of the solution used for the study (L); and *w* is the mass of biochar used (g).

2.2.3 Equilibrium and kinetics of adsorption

Equilibrium adsorption studies were carried out for 2 mg L^{-1} of DCF for 48 h. Sampling was carried out each hour for the first 12 hours and then for each 6-hour interval untilthe end of the experiment (48 h). The data was used to study the kinetics and equilibrium of adsorption for BC-PW and BC-PM. For this study, pH was kept constant at 6.5 and temperature was maintained at 25°C.

Adsorption process (adsorption rate, model, and information about adsorbent/adsorbate interaction - physisorption or chemisorption) was evaluated by Pseudo-first-order rate equation, Pseudo-second-order rate equation and Elovich equation. The theory behind each equation is given in supplementary information (SI).

2.2.4 Varying DCF concentrations and adsorption isotherms

Adsorption studies were carried out using 0.1 g of biochar microparticles concentrations varied from 0.1 mg L^{-1} to 10 mg L^{-1} (0.1, 0.25, 0.5, 1, 3, 5 and 10 mg L^{-1}). The pH was kept constant at 6.5 and temperature was maintained at 25°C. Equilibrium data were used to study the adsorption isotherm models. Langmuir, Freundlich, Temkin, and Dubinin- Radushkevich (D-R) isotherm models were used to study the adsorption process. The theory behind each model is given in supplementary information (SI)

2.2.5 pH and adsorption

Adsorption studies were carried out at different pH, 2, 6.5, 10, and 12.5 to evaluate the effect of varying pH on adsorption. In this experiment, studies were carried out using 1 mg L^{-1} of DCF prepared in milli-Q water. The pH of biochar samples was analyzed using an independent method (Nielsen et al., 2014). In short, biochar samples were added to milli-Q water containing DCF and the pH of the sample was adjusted using 1N NaOH or HCl. Adsorption experiments were carried out until reaching equilibrium and samples were analyzed for zeta potential and DCF.

2.2.6 Thermodynamics of adsorption

The thermodynamics of adsorption of DCF on BC-PW and BC-PM was evaluated by carrying out adsorption study at 10°C, 20°C, 25°C, 30°C, 40°C and 50°C with 500 µg L^{-1} of DCF. The study on the effect of temperature on adsorption will extend the knowledge of thermodynamic parameters, such as Gibbs free energy of adsorption (ΔG°), heat of the adsorption (ΔH°) and standard entropy changes (ΔS°) (Suriyanon et al., 2013). Thermodynamic equations used for these calculations are given in Equations 3 and 4:

$$\ln\left(\frac{q_e}{C_e}\right) = \frac{\Delta S^\circ}{R} - \frac{\Delta H^\circ}{RT} \quad \text{---------- (3)}$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad \text{---------- (4)}$$

Where R is the universal gas constant (8.314 J mol^{-1} K^{-1}) and T is the absolute temperature in Kelvin (K). ΔH° and ΔS° can be determined from the slope and intercept of the plots between $ln\left({}^{q_e}/_{c_e}\right)$ versus 1/T (equation 13). ΔG° can be calculated from equation 4.

2.2.7 Microparticles and adsorption

To study the effect of adsorption on biochar microparticles in comparison with raw biochar (as obtained from: millimeters to high micrometers range) adsorption studies were carried out using biochar as obtained. According to the International Union of Pure and Applied Chemistry (IUPAC) guidelines (terminology for biorelated polymers and applications (IUPAC Recommendations, 2012), microparticles are defined as particles with dimensions between 1×10^{-7} and 1×10^{-4} m. The preparation technique for microparticles is described in section 2.1. The behavior of microparticles is unique since microparticles have a larger surface-to-volume ratio than the macroscale. For further comparison activated carbon was used as a positive control for this study.

2.2.8 DCF analysis

Stock solutions of diclofenac and diclofenac-d_4 (1 g L^{-1}) were prepared in methanol and stored in amber colored bottles at 4±1 ºC, until use. All the reagents used for analysis of DCF are of analytical grade. Laser diode thermal desorption tandem mass spectroscopy (LDTD- MS/MS) was used for the analysis of DCF samples(Lonappan et al., 2016b). The instrument comprised LDTD-APCI (Laser diode thermal desorption - atmospheric pressure chemical ionization) source (LDTD T-960, Phytronix Technologies, Quebec, Canada) mounted on a TSQ Quantum access triple quadruple mass spectrometer (Thermo Scientific, Mississauga, Ontario, Canada).

3 Results and discussion

3.1 Physico-chemical properties of biochar

Scanning electron micrographs of biochar (Fig.1 in (Lonappan et al., 2017) and supplementary file) showed rough and uneven surfaces with the presence of several pores. For both C-PM and BC-

PW, as per visual image data, pores are of the dimensions of micrometers (µm). For BC-PW, pores are well arranged whereas, for BC-PM, the pores are unevenly distributed. However, these micro and macro pores facilitate the adsorption of DCF having a molecular size in A° dimension(Jung et al., 2015).

The Brunauer, Emmett, and Teller (BET) specific surface area of raw pine wood biochar (BC-PW-R) was fairly very small at 0.18 m^2 g^{-1}, whereas with microparticles of pinewood biochar (BC-PW-M) an increase in surface area (13.3 m^2 g^{-1}; about 75-fold) was observed. For raw pig manure biochar (BC-PM-R) surface area was 21.4 m^2 $g^{-1}$and with microparticles (BC-PM-M), it was increased to 43.5 m^2 g^{-1} (Nielsen et al., 2014). Thus, size reduction resulted in an increased surface area and this has been discussed in a previous study (Lonappan et al., 2016c). The surface area of activated carbon used was 900 m^2 g^{-1}.Other properties, such as ash content and moisture content which can affect adsorption properties are already given in a previous publication (Lonappan et al., 2016c). Fourier transform infra-red (FTIR) spectra was recorded for both BC-PW and BC-PM (Fig. 2 in (Lonappan et al., 2017) and supplementary file). For pinewood biochar (BC-PW), wood cellulose is a polymer rich material with hydroxyl groups and even after pyrolysis, some hydroxyl moiety remains in the sample (Chia et al., 2012) and a sharp and medium peak near 3400 cm^{-1} indicated the presence of O-H stretching in the plane with a free hydroxyl group. A medium and narrow peak at 2912 cm^{-1} showed the presence of alkanes with C-H stretching. The narrow peak at 2362 cm^{-1} was identified as the presence of nitrile group (C=N). Since BC-PW is derived from a plant source, the presence of lignin/cellulose-derived transformation products was observed (Keiluweit et al., 2010) (multiple peaks starting around 1600 cm^{-1} to 700 cm^{-1}). A strong, but narrow peak at 1584 cm^{-1} was due to aromatic ring modes which is characteristic for chars derived from lignocellulose materials, such as pinewood (softwood) (Sharma et al., 2004). A medium peak at 1373 cm^{-1} was attributed to the presence of alkane (C-H) bending. A strong peak at 1197 cm^{-1} was due to sulfonic acids with S=O stretching. A peak at 873 cm^{-1} could be due to C-H bending (1, 2, 4-trisubstituted) and a medium peak at 812 cm^{-1} signified aromatic C-H bending (trisubstituted). Moreover, a medium peak at 750 cm^{-1} was due to

aromatic C-H bend (out of the plane). The FTIR spectra of pig manure biochar (BC-PM) revealed their complex functional groups consisting of a mixture of mineral and organic matter. Generally, biochar from pig manure feedstock consists of several inorganic functional groups, such as metals (Zhang et al., 2013). For pig manure biochar, a narrow peak at 3400 cm^{-1} was identified as N-H stretch which could be due to the presence of secondary amines. The narrow peak at 2533 cm^{-1} showed the presence of mercaptans (S-H stretch). A broad and strong peak of around 1422 cm^{-1} is due to the C–C stretch (in the –ring) which confirmed the presence of aromatics. The broad and strong peak around 1114 cm^{-1} was due to C-O stretch and pointed towards the presence of carboxylic acid functional groups. Since pig manure is rich in phosphorus, a peak around 874 cm^{-1} and 563 cm^{-1} can be assigned to PO_4^{3-} group. The peak of around 712 cm^{-1} could be due to the presence of calcium and peak around 617 cm^{-1} was assigned to the presence of disulfides (S-S) stretching since pig manure is rich in minerals. Moreover, peak around 563 cm^{-1} was assigned to the presence of alkyl halides.

X-ray diffraction (XRD) analysis was conducted for BC-PW and BC-PM to study the crystallinity behaviour of the biochar (Fig. 3 in (Lonappan et al., 2017) and supplementary file). The chemical structure of the adsorbent plays a key role in the interaction between adsorbent and adsorbate and hence explains adsorption potential and mechanism. For BC-PW, distinct peaks of around 25 and 44, 2θ° angle were attributed to the presence of quartz (Zhang et al., 2015).

Particle size distribution of the prepared microparticles is given in Fig 1. The particle size distribution was well within the defined microparticles range with a diameter of less than 100 µm. The mean particle size of pig manure biochar microparticle (BC-PM) was observed to be ≈30.6 µm with 9.32 µm, 22.52 µm and 61.58 µm for D10 (particle diameter corresponding to 10% cumulative (from 0 to 100%) undersize particle size distribution), D50 (particle diameter corresponding to 50% cumulative (from 0 to 100%) undersize particle size distribution) and D90(particle diameter corresponding to 90% cumulative (from 0 to 100%) undersize particle size distribution) values, respectively.

3.2 Microparticles and adsorption

Removal efficiencies of raw biochar, biochar microparticles and activated carbon under different concentrations and at an initial hour and at equilibrium time is given in Fig.2. Adsorption studies were focused on environmentally relevant concentrations of DCF such as 500 µg $L^{-1.}$. Pinewood biochar as obtained- (BC-PW-R) showed the lowest removal efficiency for DCF at 10 mg L^{-1} and 500 µg L^{-1}. For both concentrations, the maximum removal efficiency was obtained at equilibrium which was reached after 4.5 hours. The maximum removal efficiency obtained was 40 % for BC-PW-R, whereas as with pine wood biochar microparticles (BC-PW-M), this removal efficiency was enhanced to 68% and which was obtained for 500 µg L^{-1} of DCF at equilibrium. This enhancement in removal efficiency is attributed towards the increase in surface area from 0.18 m^2 g^{-1} to 13.3 m^2 g^{-1} which provided an increase in adsorption sites. Moreover, DCF being a molecule with a negative charge; will be drawn towards the positive charges on the biochar surface. Functional groups, such as a free hydroxyl group will create positive surface radicals on biochar that will assist in the overall adsorption of DCF.

Biochar derived from pig manure exhibited higher adsorption potential and hence higher removal efficiency over pine wood biochar. Even raw pig manure biochar (as obtained form) (BC-PM –R) exhibited good removal efficiency of 61.8 % in the first hour of adsorption and which increased up to 80.8% at equilibrium at 10 mg L^{-1} DCF. Moreover, at an environmentally relevant concentration of 500 µg L^{-1}, the maximum removal efficiency obtained was 82%. With pig manure biochar microparticles (BC-PM-M), the removal efficiency was enhanced up to 98% and 99.6%, respectively at 10 mg L^{-1} and 500 µg L^{-1} and at equilibrium. This excellent removal efficiency of pig manure biochar can be attributed to its surface area: 21.4 m^2 g^{-1} for raw biochar and 43.5 m^2 g^{-1} for microparticles. The increase in removal efficiency of microparticles (BC-PM-M) was due to the availability of higher surface area for adsorption through size reduction. Moreover, FTIR analysis proved the presence of inorganic surface functional groups and metals in pig manure biochar. Presence of a variety of surface functional groups, such as halides, sulfides, carboxylic acids,

phosphates along with other organics enhanced the adsorption potential. Previous studies also reported the specific ability of pig manure-derived biochar for the removal of herbicides (Zhang et al., 2013). Furthermore, it is to be noted that at an environmentally relevant concentration, pig manure-derived biochar microparticles exhibited removal efficiency near 100% (99.6 %) at equilibrium. Commercially available activated carbon was effective at 10 mg L^{-1} DCF owing to its much larger surface area. In addition, activated carbon exhibited its potential for rapid adsorption so that equilibrium was attained in less than one hour with excellent removal efficiency (99.4 %). However, at lower concentration (500 µg L^{-1}), the removal efficiency was 95% for activated carbon even after 5 hours of contact time. For biochar microparticles derived from pig manure, the removal efficiency increased to 99.6% at equilibrium. Hence, pig manure biochar can be used as an effective adsorbent for micropollutants and as a potential replacement for activated carbon.

Apart from the enhanced surface area, several other factors influenced and increased adsorption potential/ removal efficiency of pig manure biochar in comparison with biochar derived from pine wood. Previous molecular modeling studies reported the interactions between DCF and biochar surfaces (Jung et al., 2015). In addition, depending upon the type of the adsorption, several interactions, such as p-p electron donor-acceptor interactions, hydrophobic interactions, hydrogen bonding and Van der Waal forces are involved in adsorption. The larger molecular butterfly structure of DCF(Jung et al., 2015; Lonappan et al., 2016b) maximizes the p-p interactions between the surface of biochar and DCF (Schames et al., 2004). The structure comprising one aromatic ring will be always parallel to the adsorbent and other will be perpendicular and which will enhance the interaction with biochar surface making DCF always available for biochar. In this study, biochar derived from pig manure and pinewood is rich in organic functional groups evident from the available FTIR results. The aromatic carbon will enhance the hydrophobic interactions owing to its component ratio (Chen et al., 2007; Jung et al., 2015). Also, the effect of the copious presence of non-aromatic carbon, aliphatic carbon, and other inorganic functional groups in pig manure biochar cannot be overruled. The interactions between polarizable biochar surfaces will act as electron donors and

polar aromatic DCF (electron acceptors) can also be expected (Chen et al., 2007). Hence, these interactions with aromatic groups in addition to inorganic functional groups make pig manure biochar a better adsorbent for DCF over pine wood biochar. Moreover, these enhanced interactions due to the presence of a large variety of inorganic and organic functional groups could be the reason for enhanced adsorption potential for pig manure biochar at lower concentrations in comparison with activated carbon despite lower surface area.

Co-existing ion effect also had a considerable effect on adsorption of DCF onto biochar. Zhang et al,2013 reported that presence of metals, such as Ca and Mg can enhance DCF adsorption (Zhang et al., 2013) by forming DCF- metal precipitates or complexes. Elemental and metal analysis (supplementary data) showed that Mg (mg/kg) (PM- 10111.52±78.6, PW- 143.45±10.2) and Ca (PM- 45481.88±26.8 and PW- 1753.38±14.5) concentrations are much higher in PM than PW and thus formed precipitates which in turn resulted in enhanced adsorption by BC-PM. Moreover, XPS analysis has been conducted to confirm co-existing ion effect as well as further confirm the results obtained from FTIR.

From XPS results, it is evident that the presence of other ions, such as metals enhanced the adsorption potential of BC-PM. Detailed report on XPS analysis has been provided as supplementary information. For pinewood biochar, the fit is not perfect with the function used for the asymmetry, but as expected the main peak is highly asymmetrical and its width is very small; it can be shown that the π-π* satellite has an intensity only between 2.6 and 4.6% of the total C1s intensity, it is located at around 6 eV on the high BE side of the main peak and has a width larger than 2eV. As there are no contaminants on this sample, especially oxygen, there are no peaks associated with carbon-oxygen bonds.

For pig manure biochar, O1 and C1 spectrums showed the presence of various impurities and metal oxides. Moreover, C=O and C-O bonding were predominant in these samples. 2p Ca was also observed that can directly influence the DCF adsorption through metal complexation of DCF with Ca.

3.3 Equilibrium studies and kinetic models

The equilibrium time was determined based on adsorption studies on biochar microparticles across time. Results showed that equilibrium was reached after 4.5 hours for BC-PW and after 5 hours for BC-PM. The initial rapid adsorption for both biochars facilitated a rapid adsorption to reach equilibrium. Moreover, nature and available sorption sites of adsorbents affected the adsorption equilibrium (Bhattacharya et al., 2008).

Adsorption kinetic models can predict chemical reactions, reaction rate, and particle diffusion mechanism of DCF onto biochar microparticles. However, elementary kinetic models, such as first and second order rate equations are not applicable to the adsorption system with solid surfaces, such as biochar, which are rarely homogeneous since the effects due to chemical reactions involved and the transport phenomena are often experimentally inseparable (Sparks, 1989). In this study, pseudo first-order, pseudo second-order, Elovich l and the intra-particle diffusion models were used. Results (Table 1) showed that kinetics of DCF adsorption onto biochar microparticles were correlated with the linear forms of these four models. Kinetic equations and theoretical aspects involved in the models are given in supplementary information.

For BC-PW, all the studied models exhibited a good correlation with the experimental data (correlation coefficients obtained were 0.93, 0.99 and 0.95 respectively for pseudo first-order, the pseudo-second-order and Elovich kinetic). However, even with a correlation coefficient near to unity, pseudo-second order failed to obtain a theoretical equilibrium concentration from a model which was equal or comparable with the experimentally observed equilibrium concentration. With a correlation coefficient of 0.938, calculated equilibrium concentration was comparable with experimentally observed equilibrium concentration in pseudo-first-order model. Elovich model also gave a better fitting to the experimental data with a correlation coefficient of 0.95. However, the pseudo first-order model was selected as the model for the adsorption DCF on BC-PW due to its excellent agreement with the experimental equilibrium concentration value. Hence during adsorption mass transfer from the bulk to the adsorption sites are occurred driving and the driving force is the concentration

difference (Lagergren, 1898; Smith et al., 2016). Thus, the concentration of DCF in the bulk solution played a significant role in the adsorption of DCF on to BC-PW.

For BC-PM, correlation coefficients of 0.937, 0.9915 and 0.970 were observed for pseudo first-order, pseudo second-order and Elovich kinetic model respectively. Moreover, equilibrium concentration calculated from pseudo-second order was in better agreement with the observed experimental equilibrium concentration with a percent error of less than 5%. Thus the adsorption kinetics of BC-PM was better explained by pseudo-second-order kinetic model. As a result, chemical reaction/ chemisorption can be assumed as the rate controlling step for the adsorption process(Smith et al., 2016). A chemical reaction involving the strong complexation of DCF with the active sites on the BC-PM took place during adsorption. Moreover, pseudo second-order suggests the rate of adsorption for BC-PM is dependent on the availability of the sorption sites rather than the concentration of the DCF in the bulk solution (Liu, 2008). The Elovich model gives also a better fitting to the experimental data with a correlation coefficient of 0.97 indicates that chemisorption is a dominant process during adsorption and suggesting heterogeneous adsorption (Low, 1960).

Both BC-PM and BC-PW exhibited good agreement with the intraparticle diffusion model and hence for both samples mechanism of adsorption can be explained on the basis of intraparticle diffusion. However, for intraparticle diffusion mechanism was not the rate-determining step since the intercept did not cross the origin in the IPD curve(Dural et al., 2011)(data not shown).

3.4 Adsorption isotherm modeling

The adsorption isotherm explains about the interactions between DCF and the biochar microparticles and consequently the adsorption mechanisms. Theoretical aspects regarding adsorption isotherms and the isotherm plots are given as supplementary data. Isotherm data obtained for different adsorption isotherm models for both BC-PW and BC-PM is given in Table 2. BC-PW followed the Langmuir adsorption isotherm model with R^2 =0.984 and hence homogeneous monolayer adsorption onto the surface can be expected (Tan et al., 2009). Thus no re-adsorption to

the surface is taking place during the adsorption. Temkin (R^2 = 0.92) and Dubinin- Radushkevich (D-R) (R^2=0.73) isotherm models were not linearly fitting the experimental data and thus were not considered. However, Freundlich isotherm model resulted in R^2 of 0.94. Even though, owing to higher R^2 obtained with Langmuir isotherm model and a Q_0 (µg g-1) of 526.3 which was almost equal to the Q_0 observed in actual experimental conditions, Langmuir isotherm model has been thus selected as the best fitting model. Hence, a maximum adsorption capacity of 526.3 µg g^{-1} can be observed at 500 µg L^{-1} of DCF through isotherm models. Moreover, Langmuir isotherm model rate of adsorption has been calculated as 1.39 x 10^{-3} L μg^{-1} (Kilduff et al., 1996; Tan et al., 2009).

BC-PM followed the Freundlich isotherm model (R^2 = 0.99) which involved multilayer adsorption. All the other models (Langmuir, Temkin, and D-R) resulted in poor R^2 with the experimental values (Table 1). Thus, the stronger binding sites on the surface are occupied first and that the binding strength decreased with the increasing degree of site occupancy and which reduced the adsorption with time. Hence, after an initial increase in adsorption capacity, adsorption decreased gradually due to unavailability of adsorption sites (Freundlich, 1906; Tan et al., 2009). Moreover, Freundlich isotherm model suggested the possibility of heterogeneous multilayer adsorption involving binding by both physical and chemical forces. However, the high value of n (favorability factor for heterogeneity; 1/n = 0.87) ruled out the possibility of heterogeneity in this study.

3.5 pH effect on adsorption of DCF

Effect of pH on adsorption is an important parameter to study as adsorption is being a surface controlled process. The pH of BC-PW was found to be 8.5 and 11.22 for BC-PM. Thus, negative surface charges are predominant on the surface of biochar. Fig.3 demonstrated a correlation between pH, zeta potential and the adsorption of DCF on biochar. For BC-PW and BC-PM, higher adsorption occurred at acidic pH. Moreover, BC-PW was significantly affected by pH (88.6±2 % at pH 2 to 36±3 % at pH 12.5) as compared to BC-PM (about 99.8 ± 2 % at pH 2 to 88.8 ± 2 % at pH

12.5) for removal efficiency of DCF. Furthermore for both biochars removal was always favored by acidic pH.

Ionizable micropollutants can interact with adsorbents through electrostatic attraction or repulsion, and this interaction varied depending on their pKa values (Huerta-Fontela et al., 2011). DCF is considered as a weak acid as its pKa is around 4.15 (Nam et al., 2014). For hydrophobic compounds such as DCF, the adsorption could be largely affected by the pH changes. Hence, electrostatic and specific sorbate-sorbent interactions (based on surface polarities, functional groups, organic and inorganic components of biochars)(Nielsen et al., 2014) between DCF and biochar surface do have an impact on adsorption.

The isoelectric point (pH_{IEP}) of BC-PW was calculated to be 2.45 from zeta potential - pH curve (Fig 3). Excellent removal efficiency (88.6 %) and hence adsorption was exhibited by BC-PW at acidic pH. DCF being a negative ion at acidic pH higher adsorption can be expected. Consequently, at acidic pH and even pH near to pH_{IEP}, excellent removal was obtained. This phenomenon can be explained on the basis of electrostatic attraction between negatively charged DCF and positive biochar surface. Further, removal efficiency was decreased by 60% with an increase in pH. The electrostatic interactions played a major role in the adsorption of DCF onto BC-PW microparticles. Adsorption of DCF onto BC-PW was mainly due to physical forces of attraction which may include electrostatic interactions, Van der Waals forces, and hydrophobic interactions. In addition, both the biochar and DCF are hydrophobic in nature and this factor might have facilitated the adsorption and favorable pH. Excellent adsorption near to (pH_{IEP}) is suggested the possibility of other mechanisms involved in the adsorption process. This can be attributed to H- bonding possibility with oxygen-containing groups, such as OH^- on the surface of biochar (Fang et al., 2014; Nam et al., 2015) along with hydrogen bonding with other aromatic functional groups, such as aromatic C-H which are observed on the surface of biochar.

The isoelectric point (pH_{IEP}) of BC-PM was calculated to be 2.15 from zeta potential - pH curve. For BC-PM, the adsorption was also pH dependent, the maximum removal efficiency (99.8%) was

observed at pH of 2; while it decreased to 88.8% at pH 12.5. This decrease could be due to the changes in the surface charge and hence the negative surface of biochar repelled DCF anion. However, in comparison with BC-PW, the drastic decrease in removal efficiency was minimum and even at unfavorable pH of 12.5; 88.8% of removal efficiency was observed. Physical processes, such as electrostatic interactions are not the major process involved in the adsorption DCF onto BC-PM. The removal process can be explained on the basis of electrostatic interactions, H- bonding, hydrophobic effects, and π–π EDA interactions. The presence of oxygen-containing functional groups, such as carboxylic acids facilitated the adsorption through H- bonding irrespective of pH (Fang et al., 2014). Moreover, polar functional groups, such as hydroxyl and amine groups exhibited electron-withdrawing effect at basic pH values(Ji et al., 2010) and these groups can interact with aromatic rings (π electron acceptors) in BC-PM. Minerals and inorganic functional groups, such as disulfides and halides are present in BC-PM and has positively affected the adsorption, irrespective of the pH.

3.6 Thermodynamics of DCF adsorption

Adsorption studies were carried out at various temperatures: 10°C, 20°C, 25°C, 30°C, 40°C and 50°C. Thermodynamic parameters were calculated according to equations (13) and (14). Different behaviors were observed for BC-PW and BC-PM with adsorption along with temperature (Table 3). In the liquid phase adsorption, the adsorption of adsorbate occurred while solvent species were removed from the sorption sites. Displacement enthalpies are the key factors in this process as they balance different weak interactions between adsorbent, adsorbate, and solvent.

The thermodynamic factors affecting the adsorption process are summarized in Table 3. For BC-PW, a decrease in adsorption capacity (μg g^{-1}) was observed along with an increase in temperature. An adsorption capacity of 141.82 μg g^{-1} was observed at 10° C (283K) and which decreased along with an increase in temperature to 75.34 μg g^{-1} at 50° C (323K) under equilibrium conditions. Previously, similar results were observed for diclofenac adsorption on Isabel grape bagasse

(Antunes et al., 2012) and for the removal of other pharmaceuticals on carbon black (Cuerda-Correa et al., 2010). In these studies, authors explained this phenomenon based on two processes: the energy exchange between sorbate, sorbent and the solvent and the solubility of pharmaceuticals. Elevated temperatures can increase the solubility of the pharmaceutical; hence in a better soluble condition, pharmaceuticals will have a higher affinity towards the solvent than the adsorbent. In addition, along with temperature, vapor pressure also increased and density of the adsorbate decreased which will decrease the adsorption as a result of decreased interactions between DCF and biochar surface (BC-PW). For BC-PW, adsorption was endothermic ($\Delta H°$ =1.130 KJ mol^{-1}) Moreover, 1.130 KJ mol^{-1} for $\Delta H°$ indicated that the process is physisorption since the adsorption energies for physisorption are lower than 40 KJ mol^{-1}. A negative value of $\Delta S°$ (-4.40 J mol^{-1} K^{-1}) suggested that randomness at the solid –solution interface decreased during the adsorption. The possible reason could be the formation of more than one layer of adsorption (Antunes et al., 2012). Also, the total process was non-spontaneous ($\Delta G°$= positive). A similar behavior was observed in a previous study as well for dye adsorption onto activated bentonite(Özcan & Özcan, 2004). For DCF adsorption, a similar behaviour was observed with adsorption of DCF on to Isabel grape bagasse (*Vitis labrusca*)(Antunes et al., 2012). The positive value and thus non-spontaneity of the process could be due to the presence of energy barrier which hindered the adsorption process. This could be due to the repulsive electric charges (negative) (Antunes et al., 2012; Özcan & Özcan, 2004) which were present on the surface of biochar. Adsorption of DCF on BC-PW was highly pH dependent (section 3.5) and electrostatic attraction forces played a major role in the adsorption and hence thermodynamic behaviour of DCF adsorption onto BC-PW is in good agreement with the observations made using pH variable study.

A completely different behavior was obtained for BC-PM, a small increase in adsorption capacity (µg g^{-1}) was observed along with an increase in temperature. An adsorption capacity of 236.53 µg g^{-1} was observed at 10° C (283K) and which increased along with an increase in temperature to 247 µg g^{-1} at 50° C (323K). Thus, the effect of temperature on adsorption was minimal. This increase can

be attributed to increased surface coverage of DCF at higher temperature due to the expansion and creation of new active sites on BC-PM. The larger size of DCF and the possible presence of metal oxide catalysts (creates active sites at a higher temperature, identified by FTIR) in BC-PM could be the reason for slightly better adsorption at higher temperatures. For BC-PM, a value of $\Delta H° = -3.970$ KJ mol^{-1} suggests adsorption process as exothermic. A positive value of $\Delta S°$ (16.13) suggested that randomness at the solid-liquid interface increased during the adsorption. The whole process was spontaneous; indicated by a negative $\Delta G°$ value. Similar results for spontaneity have been reported for adsorption of anti-inflammatory pharmaceutical naproxen on activated carbon (Önal et al., 2007)

4. Conclusions

The adsorption of DCF onto BC-PM was found to be effective over BC-PW. About 99.6% removal efficiency was observed for BC-PM at an environmentally relevant concentration of 500 µg L^{-1}. For BC-PW, adsorption was explained by pseudo first-order model whereas for BC-PM by pseudo second-order model. For both adsorbents, intraparticle diffusion was found to be the major mechanism explaining the adsorption behavior. BC-PW adsorption was largely affected by the pH, whereas pH dependency of adsorption of BC-PM was minimal. Thermodynamic behavior of both adsorbents suggested adsorption of DCF on BC-PW was endothermic while exothermic for BC-PM.

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Table 1: Kinetic parameters for the adsorption of DCF onto biochar microparticles, BC-PW and BC-PM

Micro-particle sample	q_e, experimental (μg g^{-1})	Pseudo-first order model			Pseudo-second order model			Elovich model			Intraparticle diffusion model	
		q_e, cal. (μg g^{-1})	k_1 (min^{-1})	R^2	q_e, cal. (μg g^{-1})	K_2 (g μg^{-1} min^{-1})	R^2	α (μg g^{-1} min^{-1})	β (g μg^{-1})	R^2	k_{id} (μg $g^{-1}min^{-1/2}$)	R^2
BC-PW	331	339.7	0.0133	0.938	400	3.82×10^{-5}	0.992	13.87	0.0117	0.9597	38.40	0.982
BC-PM	955	480.83	0.0175	0.937	1000	4.40×10^{-5}	0.991	5.07×10^{6}	0.0179	0.970	674.5	0.977

*BC-PW: Pine wood biochar microparticles, BC-PM: Pig manure biochar microparticles

q_e and q_t are adsorbed (μg g^{-1}) DCF on biochar at equilibrium and at time t (min) respectively and k_1 is the rate constant of adsorption (min^{-1}). k_2 (g μg^{-1} min^{-1}) is the adsorption rate constant of pseudo-second-order adsorption process. α is the initial sorption rate (μg g^{-1} min^{-1}) and β is the desorption constant (g μg^{-1}). k_{id} (μg $g^{-1}min^{-1/2}$) is the intra-particle diffusion rate constant obtained from the intercept.

Table 2: Isotherm parameters adsorption of DCF onto biochar microparticles, BC-PW and BC-PM at equilibrium conditions

Micro-particle sample	Langmuir isotherm model			Freundlich isotherm model			Temkin isotherm model				Dubinin- Radushkevich (D-R) isotherm model			
	Q_0 (µg g^{-1})	K_L ($L\mu g^{-1}$)	R^2	K_F (µg g^{-1})	n	R^2	A_T ($L\mu g^{-1}$)	b_T	B	R^2	q_m (µg g^{-1})	β (mol2 kJ -2)	E (kJ mol^{-1})	R^2
BC-PW	526.3	$1.39x10^{-3}$	0.984	6.774	1.919	0.939	0.027	27.63	89.64	0.924	236.27	0.0005	0.0316	0.735
BC-PM	12500	$8.47X10^{-4}$	0.891	16.61	1.145	0.995	0.035	2.023	1224.4	0.878	2184.18	0.0002	0.050	0.773

*BC-PW: pine wood biochar microparticles, BC-PM: Pig manure biochar microparticles

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Q_o and K_L are Langmuir constants for adsorption capacity and rate of adsorption, respectively. K_F is the adsorption capacity of the adsorbent and n is the favourability factor of the adsorption. b_T is the Temkin constant related to heat of sorption (J mol^{-1}) and A_T is the Temkin isotherm constant (L g^{-1}). q_m is the amount of DCF adsorbed onto per unit dosage of biochar (µg g^{-1}), q_m is the theoretical monolayer sorption capacity (µg g^{-1}) and β is the constant of the sorption energy (mol^2 J^{-2})

Table 3: Thermodynamic parameters for the adsorption of DCF onto biochar microparticles, BC-PW and BC-PM

Adsorbents	Temperature (K)	Removal (%)	ΔG° (kJ/mol)	ΔH° (kJ/mol)	ΔS° (J/mol K)
	283	56.73	2.376		
	293	53.57	2.420		
BC-PW	298	52.93	2.442	1.130	-4.404
	303	50.10	2.464		
	313	47.27	2.508		
	323	30.13	2.552		
	283	94.61	-8.534		
	293	95.83	-8.696		
	298	97.04	-8.776	-3.970	16.13
BC-PM	303	98.68	-8.857		
	313	98.74	-9.018		
	323	98.80	-9.180		

*BC-PW : pine wood biochar microparticles, BC-PM : Pig manure biochar microparticles

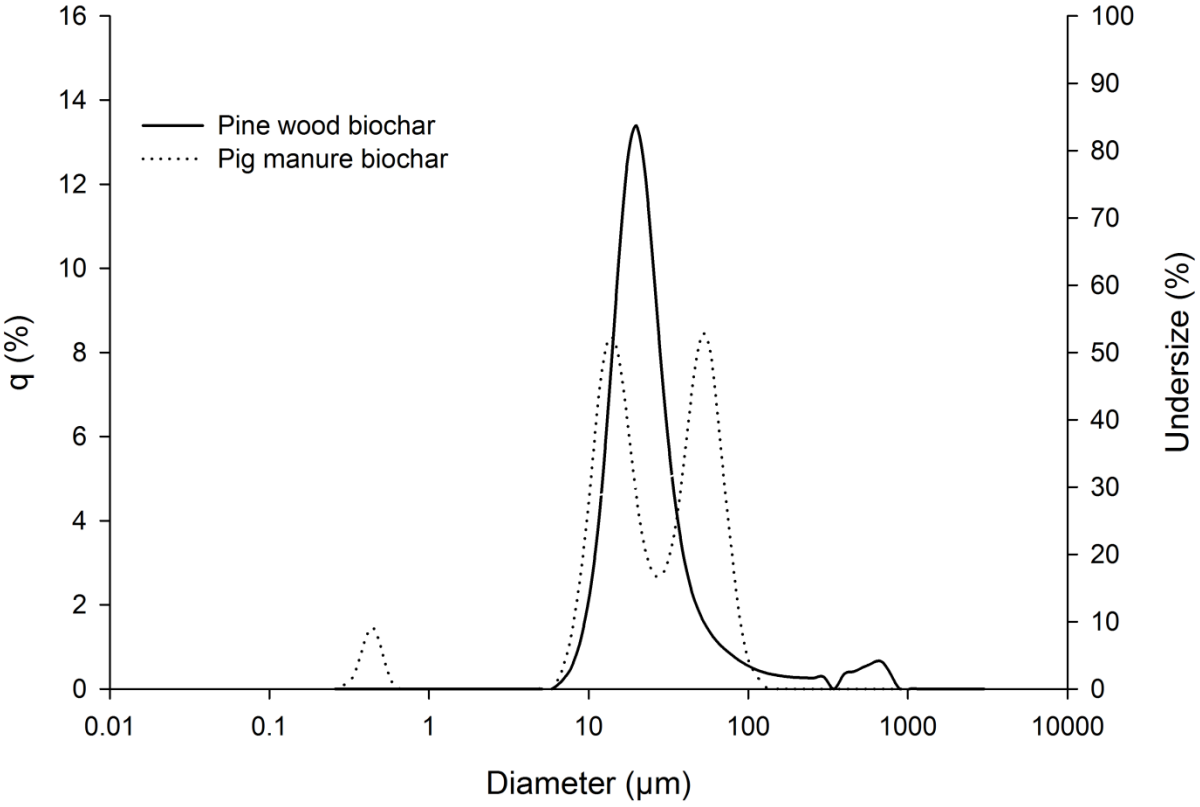

Figure 1: Particle size distribution of biochar microparticles

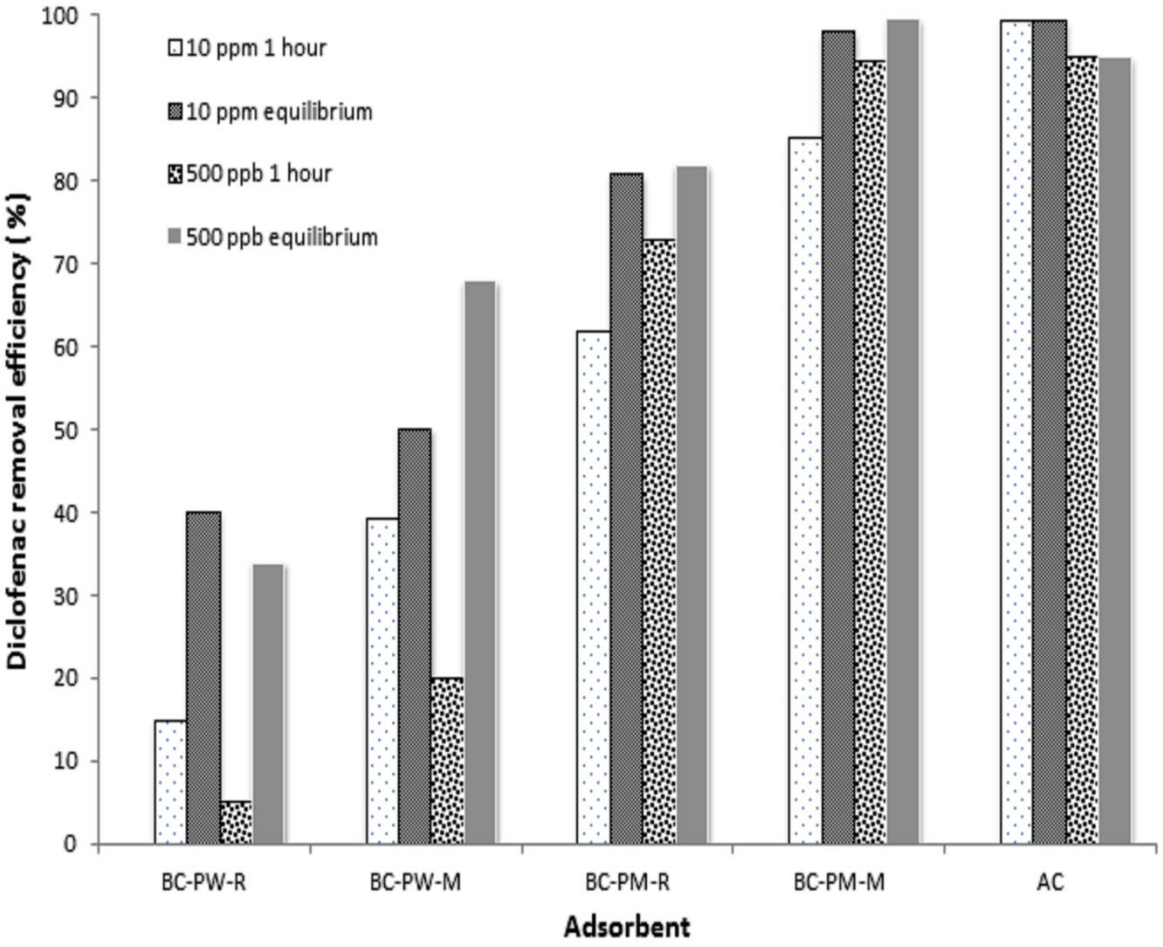

Figure 2: Biochar microparticles and diclofenac removal efficiency (BC-PW-R: raw pinewood biochar, BC-PW-M : pinewood biochar microparticles, BC-PM-R : raw pig manure biochar, BC-PM-M : pig manure biochar microparticles, AC : activated carbon)

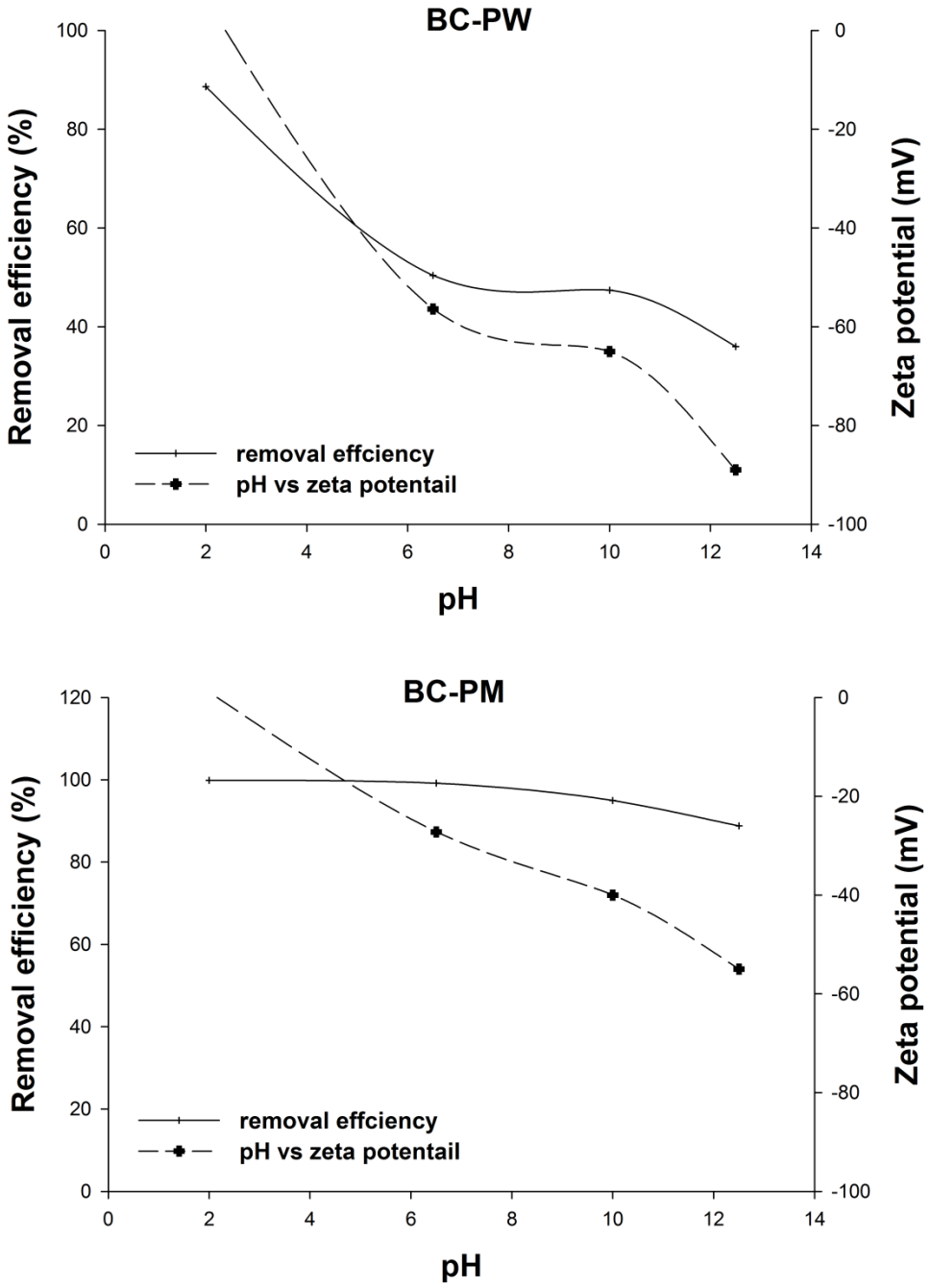

Figure 3: Effect of pH and zeta potential on removal efficiency of diclofenac (1 mg L-1 concentration); BC-PW: pine wood biochar, BC-PM: pig manure biochar

PART 3
ADSORPTION OF DICLOFENAC ONTO DIFFERENT BIOCHAR MICROPARTICLES: DATASET - CHARACTERIZATION AND DOSAGE OF BIOCHAR

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DATA IN BRIEF 16 (2018), 460 - 465

RÉSUMÉ

De par leur distribution importante dans les ressources en eau et leur toxicité, les composés pharmaceutiques et les produits de soins personnels deviennent une préoccupation émergente à travers le monde. L'application de résidus/déchets pour le traitement des eaux peut être une bonne stratégie de gestion et de valorisation des déchets. Dans ce document, le jeu de données fourni de l'information sur l'application du biochar pour l'enlèvement du diclofenac, un contaminant émergent retrouvé dans les matrices d'eau. Les données présentés dans ce document sont une prolongation d'un l'article de recherche expliquant les mécanismes d'adsorption du diclofenac sur le biochar .Cet article de données fourni de l'information générale sur les particularités de surface des biochar de bois de pin et de fumier de cochons mesurées avec un SEM et un FTIR. Ce jeu de données fourni aussi de l'information sur les profiles XRD des biochars de bois de pin et de fumier de cochon. De plus, différentes concentration de biochars ont été utilisées au cours de cette étude pour réduire une concentration fixe de diclofenac et les résultats sont présentés dans ce jeu de données.

Mots clés : Adsorption; diclofenac; biochar; caractérisation

ABSTRACT

Due to its wide occurrence in water resources and toxicity, pharmaceuticals and personal care products are becoming an emerging concern throughout the world. Application of residual/waste materials for water remediation can be a good strategy in waste management as well as in waste valorization. Herein, this dataset provides information on biochar application for the removal of emerging contaminant, diclofenac from water matrices. The data presented here is an extension of the research article explaining the mechanisms of adsorption diclofenac on biochars [1]. This data article provides general information on the surface features of pine wood and pig manure biochar with the help of SEM and FTIR data. This dataset also provides information on XRD profiles of pine wood and pig manure biochars. In addition, different amounts of biochars were used to study the removal of a fixed concentration of diclofenac and the data is provided with this data set.

Keywords: Adsorption; diclofenac; biochar; characterization

Specifications

Subject area	**Chemistry/Chemical engineering**
More specific subject area	Adsorption, Surface Chemistry, Environmental Engineering
Type of data	Table, image (XRD, SEM)), text file, figure(FTIR)
How data was acquired	SEM: Zeiss Evo®50 Smart SEM FTIR: Perkin Elmer, Spectrum RXI, FT-IR instrument fitted with lithium tantalate ($LiTaO_3$) detector XRD: Panalytical Empyrean XRD with monochromatized CuK alfa radiation (1.5418 A). LDTD-MS/MS: Concentrations of diclofenac was measured using LDTD-APCI (atmospheric pressure chemical ionization) source (LDTD T-960, Phytronix Technologies, Quebec, Canada) mounted on a TSQ Quantum access triple quadruple mass spectrometer (Thermo Scientific, Mississauga, Ontario, Canada)
Data format	Pre-processed and analyzed
Experimental factors	Biochar samples (from pinewood and pig manure) were grounded to obtain microparticles and the data here is given is for characterization of biochar. Moreover, data for dosage effect of biochar on adsorption for diclofenac is given.
Experimental features	Characterization data of biochar microparticles obtained from SEM, XRD, and FTIR are given. Adsorption studies were carried out for the removal of diclofenac using biochar microparticles. Various biochar dosages ranging from 1g $L^{-1}$to 20 g L^{-1}were tested.
Data source location	Bioprocessing and NanoEnzyme Formulation Facility (BANEFF), INRS- ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

Data accessibility	Data presented in this article
Related research article	The associated research article related to this data set is [1]

Value of the data

- Characterization data for biochar derived from two different feedstock (pine wood and pig manure) are given.
- Dataset provides an insight to the surface features of biochar.
- Dataset gives information on the adsorption capacity of biochar for emerging contaminant diclofenac.
- Dataset would be useful to identify the dosage effect of biochar on the adsorption of diclofenac.

1. Data

The dataset comprises characterization as well as experimental data. Figure 1 presents the scanning electron micrographs (SEM) of pine wood and pig manure biochar microparticles. Figure 2 presents Fourier-transform infrared spectroscopy (FTIR) images of biochar microparticles. Figure 3 presents X-ray Diffraction (XRD) images of biochar microparticles. Table 1shows the effect of adsorbent dosage on the removal of diclofenac and removal efficiency.

2. Experimental Design, Materials and Methods

2.1 Biochar microparticle preparation

Two types of biochars were prepared from pinewood and pig manure and named as BC-PW and BC-PM, respectively. Preparation of biochar and microparticles are explained elsewhere [1, 2].

2.2 Characterization of biochar microparticles

Scanning electron micrographs of the biochar microparticles are recorded using Zeiss Evo®50 Smart SEM system. FTIR spectra of the adsorbents were recorded using Perkin Elmer, Spectrum RXI, FT-IR instrument fitted with lithium tantalate ($LiTaO_3$) detector. XRD spectra of the adsorbents were recorded using Panalytical Empyrean XRD fitted with monochromatized CuK alfa radiation (1.5418 A).

2.3 Adsorption studies

Adsorption studies were carried out using 50 mg (1 g L-1) , 0.1g (2 g L-1), 0.3g (6 g L-1), 0.5g (10 g L-1), 0.7g (14 g L-1) and 1g (20 g L-1) of biochar samples with 50 mL of 500 µg L-1 of diclofenac (DCF). Batch adsorption studies were carried out in an INFORS HT - multitron standard shaking incubator (INFORS, Mississauga, Canada). Experimental conditions are as follows - shaking speed: 200 rpm; temperature: 25 ±1°C, pH: 6.5, centrifugation (after adsorption studies): at 11600 x g for 10 minutes in a MiniSpin® plus centrifuge. The supernatant was analyzed for remaining DCF using LDTD-MS/MS [3].

It was observed that for both BC-PW and BC-PM, increasing the adsorbent dosage considerably enhanced the removal efficiency. BC-PM possessed better adsorbent properties than BC-PW and showed higher potential for the removal of DCF compared to BC-PW. With a dosage of 2 g L-1, BC-PM achieved a removal efficiency of 95.87 % and above 2 g L-1 dosage level, BC-PM always achieved nearly 100% removal efficiency. For BC-PW, removal efficiency increased from 43 % to 98.8% with a dosage varying from 2 to 20 g L-1. However, the adsorption amount (µg g-1) on biochar decreased with increase in adsorbent dosage. This observation can be explained as a consequence of partial aggregation of biochar at higher concentrations of biochar which will decrease the active sites on the surface of biochar [4, 5]. Adsorbent dosage experiment was carried

out at equilibrium time and samples were drawn. In the case of BC-PW, the complete removal might have been obtained during any time of the adsorption. Therefore, adsorption amount cannot be considered as the equilibrium adsorption capacity of the biochar BC-PM. As shown in Fig. 1, porous structure of biochars probably had a positive effect on the adsorption of DCF [6]. Moreover, as shown in Fig 2, both biochars are rich in surface functional groups which in turn can facilitate the adsorption.

Acknowledgements

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Table 1: Effect of adsorbent dosage on adsorption amount and removal efficiency

Adsorbent dosage (g L^{-1})	Removal efficiency (%)		Adsorption amount on biochar (µg g^{-1})	
	BC-PW	BC-PM	BC-PW	BC-PM
2	42.9	95.8	107.4	239.6
6	83.1	100	69.3	-
10	93.6	100	46.8	-
14	96.5	100	34.4	-
20	98.8	100	24.7	-

*BC-PW: Pinewood biochar microparticles, BC-PM: Pig manure biochar microparticles

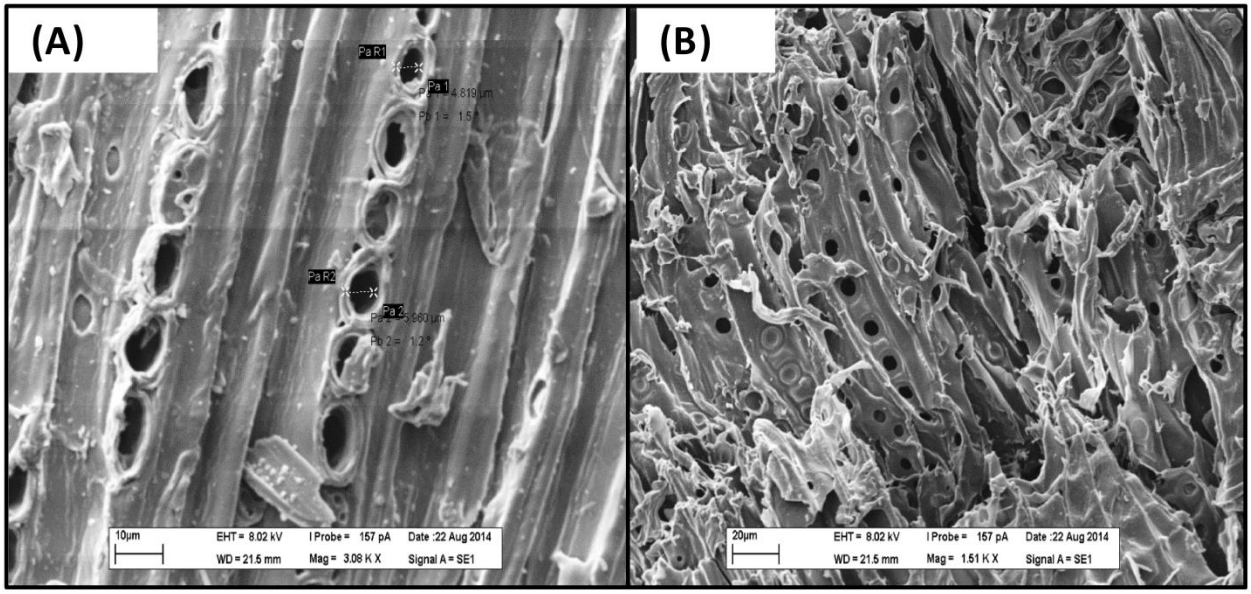

Figure 1: Scanning electron micrographs of biochar: (A) Pinewood biochar (BC-PW), (B) Pig Manure biochar (BC-PM)

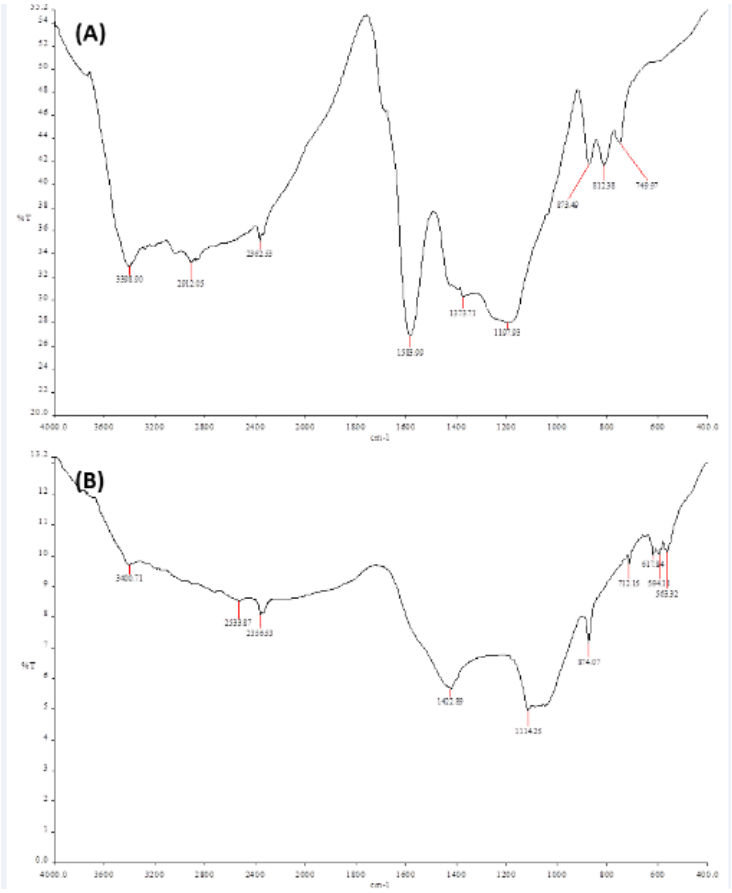

Figure 2: Fourier transform infra-red spectra and of biochar; BC-PW: pine wood biochar, BC-PM: pig manure biochar

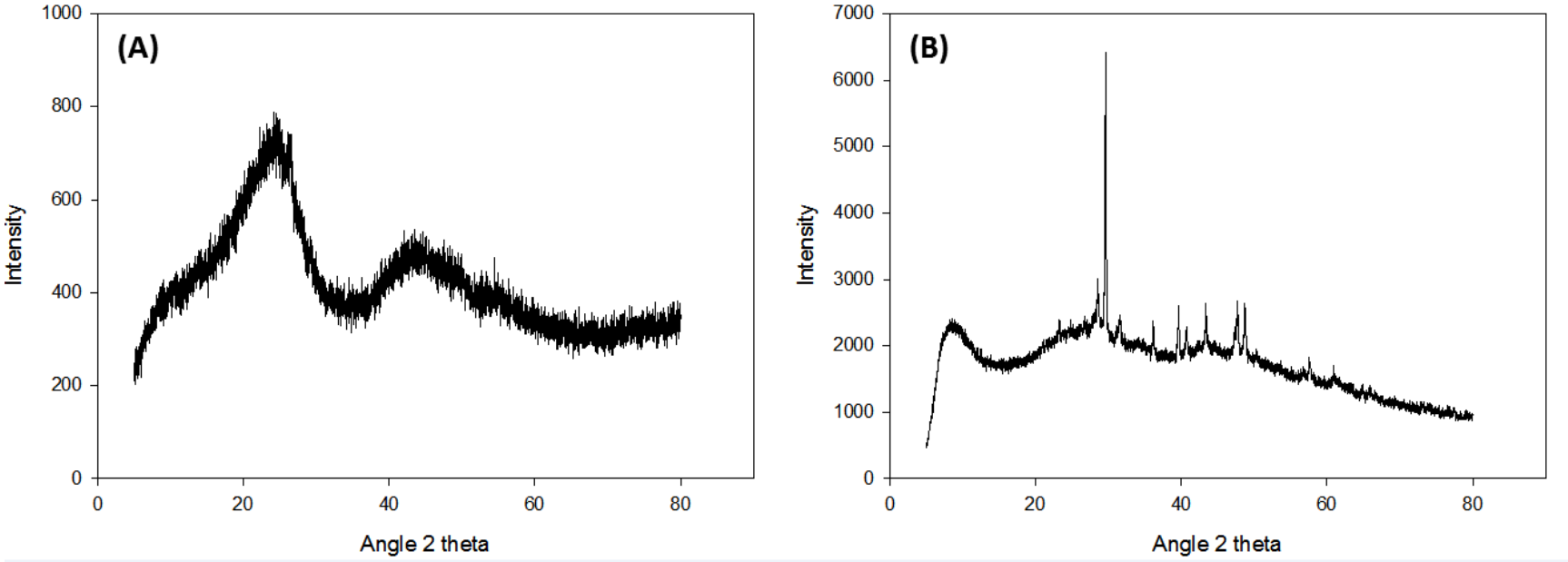

Figure 3: X-ray diffraction curve of biochar; BC-PW: pine wood biochar, BC-PM: pig manure biochar

PART 4

DEVELOPMENT OF BIOCHAR BASED GREEN FUNCTIONAL MATERIALS USING ORGANIC ACIDS FOR THE REMOVAL OF DICLOFENAC

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JOURNAL OF HAZARDOUS MATERIALS (UNDER REVIEW)

RÉSUMÉ

Les groupes fonctionnels de surface sur le biochar sont un des caractères clé pour déterminer les propriétés des biochars et leurs applications potentielles. Les études précédentes ont étudié l'amélioration des propriétés du biochar durant la production (pyrolyse) ou par activation en utilisant des acides minérales. Dans cette étude, les biochar obtenus de différentes sources, telles que le bois de pin, le fumier de porc et les coquilles d'amande, ont été fonctionnalisés en utilisant des acides organiques (acide acétique, acide citrique, acide tartrique, acide oxalique et acide malique). Deux méthodes de fonctionnalisation ont été comparées avec différentes concentrations d'acides organiques. Une par mélange d'une durée de 12h à température pièce et l'autre en chauffant à 121°C à l'autoclave pendant 20 min. Pour le biochar de bois de pin, l'acide citrique et l'acide acétique ont présenté la meilleure efficacité et l'acide citrique s'est montré plus efficace pour greffer des groupements –COOH (amélioration de 40%). Les acides tartrique, oxalique et citrique se sont montré efficace basé sur les données de FTIR avec le biochar de fumier de porc. Cependant, l'effet des acides est minimal pour la fonctionnalisation. Similairement, les acides citrique et oxalique ont été efficaces avec le biochar de coquille d'amande. Un simple mélange au cours d'une période prolongée s'est avéré plus efficace que l'autoclavage à haute température pour une courte période. La fonctionnalisation à l'acide citrique a augmenté le nombre total de groupes fonctionnels par 23.6%, 10.2% et 26.2% pour les biochars de bois de pin, de fumier de porc et de coquille d'amande, respectivement. Les biochar fonctionnalisé avec l'acide citrique ont présentés un enlèvement supérieur du diclofenac à des concentrations environnementales appropriées (500 µg L^{-1}). Pour le biochar de bois de pin, l'enlèvement du diclofenac est passé de 65% à 80%. Pour le biochar de coquille d'amande, sous des conditions similaires, l'enlèvement du diclofenac est passé de 58% à 84%.

Mots clés : Biochar; acide organique; fonctionnalisation; adsorption; diclofenac; acide carboxylique

ABSTRACT

Surface functional groups on biochars are one of the key features which determine biochar properties and their potential applications. Previous studies investigated improving properties of biochar during the production (pyrolysis) or by activation using minerals acids. In this study, biochars obtained from various feedstocks, such as pine wood, pig manure, and almond shell were functionalized using organic acids, such as acetic acid, citric acid, tartaric acid, oxalic acid and malic acid. Two methods of shaking overnight at room temperature and heating at 121°C for 20 minutes using an autoclave were tested and compared along with various concentrations of organic acids. For pinewood biochar, citric and acetic acids were found to be effective and citric acid was more efficient in terms of grafting –COOH groups (40% improvement). Tartaric, oxalic and citric acids were found to be effective based on FTIR data for pig manure biochar; however, the effect of acids was minimal on functionalization. Similarly, citric and oxalic acids were found to be effective for almond shell biochar. Simple shaking over a long period was found to effective over autoclaving at high temperature for short period. Citric acid functionalization increased total acidic functional groups by 23.6 %, 10.2 %, and 26.2 %, respectively for pine wood, pig manure and almond shell. Citric acid functionalized biochars showed higher removal of the environmentally relevant concentration (500 µg L^{-1}) of emerging contaminant, diclofenac. For pinewood biochar, at equilibrium diclofenac removal increased from 65 to 80 %. For almond shell biochar, under similar conditions, the diclofenac removal efficiency increased from 58 to 84 %.

Keywords: Biochar; Organic acids, Functionalization; Adsorption; Diclofenac; Carboxylic acid

1. Introduction

Biochar is a carbon-rich material produced through pyrolysis of various feedstock ranging from agricultural residues to organic waste materials. For the past decades, application of biochar as a method for mitigating global warming is steadily increasing (Woolf et al., 2010). Moreover, preparation of biochar from waste materials is an excellent method of waste management and valorization (Lonappan et al., 2016b). The diversity of biochar application ranges from carbon sequestration, soil improvement and decontamination of various pollutants from the soil as well as drinking/wastewater. Physico-chemical properties of biochar are the key factors while choosing its applicability and which depends on pyrolysis conditions and the feedstock selection (Méndez et al., 2009; Mohan et al., 2014; Sharma et al., 2004). The mechanism and processes behind the formation of various functional groups during carbonization/pyrolysis is still being debated. However, studies have suggested that carbonization is a dehydroxylation/dehydrogenation and aromatization process involving the cleavage of O-alkylated carbons and anomeric O-C-O carbons along with the formation of fused-ring aromatic structures and aromatic C-O groups(Li et al., 2013). High-temperature pyrolyzed biochar is known to have higher number of surface functional groups (Jindo et al., 2014; Li et al., 2013). It is rather difficult to engineer pyrolysis for specific functional groups on the biochar surface than focusing on obtaining a better surface area and porosity. Thus, modifying biochar after production can be an effective method for grafting specific surface functional groups on biochar surface.

Achievement of desired physicochemical properties, such as high porosity, high surface area, and specific surface functional groups are often laden with challenges. Therefore, several studies proposed a pre-treatment/activation/ functionalization steps of biochar before the desired application to enhance biochar effectiveness (Liu et al., 2015; Lonappan et al., 2017; Qian et al., 2007; Tan et al., 2009; Yan et al., 2013). Majority of these studies focused on increasing the surface area during activation, either more functional groups are added to the surface or certain functional groups will be removed (A and Hegde, 2015; Mohamad Nor et al., 2013). In general, chemical activation involves

mineral acids raising toxicological and environmental concerns. Activation is generally energy and cost-intensive. In addition, application of hazardous materials, such as mineral acids (for activation), which is the most commonly used, are challenged based on the “green chemistry principles” since biochar was already considered as a sustainable material(Liu et al., 2015; Naghdi et al., 2017). Application of organic acids can be an effective method to graft specific functional groups, such as carboxylic acid (-COOH) groups on biochar surface. Organic acids are produced by the catabolism of amino acids and are intermediates in metabolic pathways. Due to their biological origin, the organic acids can serve as green and sustainable material (Fischer and Bipp, 2005; Khan and Iqbal, 2016; Sauer et al., 2008).

It is hypothesized that carboxylic acids can functionalize the biochar surface. Carboxyl functional groups are known to be effective in adsorbing/stabilizing several contaminants, such as heavy metals (Uchimiya et al., 2012), pharmaceuticals (Guedidi et al., 2017) etc. Moreover, apart from adsorption, biochar with the copious presence of carboxylic groups on the surface can be effectively used forvarious other applications including catalysis, biochar supported nanostructures, energy storage etc. (Liu et al., 2015). In this study, biochar produced from feedstock, such as pine wood, pig manure, and almond shell were functionalized using acetic acid (CH_3COOH), citric acid ($C_6H_8O_7$), tartaric acid ($C_4H_6O_6$), oxalic acid ($C_2H_2O_4$) and malic acid ($C_4H_6O_5$). Two functionalization methods, simple shaking overnight at room temperature and autoclaving (at 121°C and 15 psi for 20 minutes) were tested and compared for the effectiveness of functionalization in terms of grafting biochar surface with –COOH groups. Various concentrations (molarities) of organic acids were also tested. Functionalization using organic acids followed several principles of green chemistry including prevention; less hazardous chemical syntheses; designing safer chemicals; safer solvents and auxiliaries and the use of renewable feedstocks. Functionalized biochar(s) obtained under optimal conditions were tested for the adsorption of micropollutant, diclofenac (DCF) and the effectiveness of removal (adsorption) was studied based on the –COOH group attached to the biochar surface. Diclofenac was selected as the model contaminant since it is one of the top 20 generic molecules

sold worldwide and considered an emerging contaminant present in wastewater (Lonappan et al., 2016a). To the best of our knowledge, the organic acids have never been used for the functionalization of biochars and hence entail the novelty element of the present study.

2. Materials and Methods

2.1 Materials

Pinewood and almond shell biochar samples were obtained from Pyrovac Inc. (Quebec, Canada). Pinewood biochar (BC-PW) was derived from pine white wood (80% v/v) spruce and fir (20% v/v) and was produced at 525±1°C under atmospheric pressure by increasing the temperature of biomass at the rate of 25 °C min^{-1} in the presence of nitrogen. Almond shell biochar (BC-AS) was produced from almond shells through pyrolysis under atmospheric pressure at 520±1°C for 4h. Pig manure biochar (BC-PM) was a gift sample from Research and Development Institute for Agri-Environment" (IRDA), Quebec, Canada. BC-PM was derived from the solid fraction of pig slurry and prepared at 400±1 °C for 2 h. All the biochars were ground and sieved to make biochar microparticles of less than 75 µm and the detailed procedure was given in previous publications (Lonappan et al., 2016b; Lonappan et al., 2017). This pre-treatment procedure produced compact, almost uniformly sized (distributed) biochar with an increased surface area.

Acetic acid, citric acid, tartaric acid, oxalic acid and malic acid was obtained from Fisher Scientific (Ottawa, ON, Canada). Diclofenac sodium salt (98%; CAS 15307-79-6) was purchased from Fisher Scientific (Ottawa, ON, Canada) and internal standard (IS), diclofenac-d_4 was obtained from C/D/N Isotopes Inc. (Montreal, QC, Canada). Experiments were carried out in Milli-Q water and prepared using Milli-Q/Milli-RO Millipore system (Milford, MA, USA).

2.2 Methods

2.2.1 Functionalization of biochar

Various concentrations of organic acids were tested for effective functionalization of biochars. The concentrations used for various acids are given in Table 1. The solubility of the acid salts at ambient temperature was the major criterion while selecting the molarities.

Two methods were employed for the functionalization of biochars:shaking at room temperature and autoclaving. In the shaking method, 2 g biochar was mixed with 50 mL of respective organic acid (with specific concentration -dissolved in milliQ water) and mixed for 24 hours in an INFORS HT - multitron standard shaking incubator (INFORS, Mississauga, Canada) at 150 rpm. In the autoclave method, 50 mL organic acid was mixed with 2 g of biochar and vortexed for 5 minutes. Later, the mixture was autoclaved at 121±1 C and, 15 psi for 20 minutes (Heidolph™ Tuttnauer™ Vertical Top-Loading Autoclave). After autoclaving, the biochar-acid mixture was cooled to room temperature for 15 minutes.

The biochar-acid mixture obtained after both procedures was centrifuged at 3000 *x* g for 15 minutes. The excess acid was removed, and the biochar was washed using 15 mL milli-Q water (vortexing milli-Q water-biochar mixture). This procedure was repeated three times to ensure the complete removal of organic acids. After the washing procedure, the biochar was dried in a hot air oven (60± 1 C) overnight. The obtained biochar was subjected to characterization and was used for the adsorption of DCF.

2.2.2 Characterization of functionalized biochar

Particle size distribution of raw and biochar microparticles was measured using Horiba particle size analyzer (LA-950 Laser Particle Size Analyzer, Horiba, Japan).

Scanning electron micrographs of the functionalized biochars were obtained from EVO® 50 smart scanning electron microscope (SEM) (Zeiss, Germany). Surface functional groups of functionalized biochars were identified using Nicolet iS50 FT-IR Spectrometer (Thermo Scientific, USA). Elemental composition (C, H, N, and S) of functionalized biochars were quantified using an elemental analyzer (TruSpec® Micro-CHN, Michigan, USA). The oxygen (O) content (approximately) was calculated by subtracting C, N, H, and S contents from the total biochar mass. The surface charge properties of biochars were measured by zeta potential measurements, using a Nano-ZS Zetasizer (Malvern Instruments Inc., UK) in de-ionized water. Samples with suitable FTIR peaks corresponding to –COOH groups (Table 3) were subjected to acid-base titration to find out total acidity capacity (TAC) and the experiment was carried out as described by (Chen and Wu, 2004; Hu et al., 2001) and the results are presented in Table 3 in $mmolg^{-1}$ of biochar. Initially, 50 mg of raw and acid treated biochars were shaken with 25 mL, 0.01 M NaOH for 2 days in a 50-mL sealed Eppendorf tubes. The excess NaOH left in the solution was determined by titrating against 0.01M HCl.

2.2.3 Batch adsorption studies using functionalized biochars

Batch adsorption studies were carried out in 125 mL conical flasks with 50 mL of DCF solution in an INFORS HT - multitron standard shaking incubator (INFORS, Mississauga, Canada). Agitation speed was always set at 200 rpm. The temperature was set to be 25 ±1°C and the pH was set at 6.5 (DCF solution). About 0.1 g of biochar was used for the adsorption of DCF. Concentrations of 500 µg L^{-1} and 10 mg L^{-1} of DCF were used and the biochar-DCF mixture was shaken for 5 h. Afterwards, the biochar was separated from the mixture by centrifugation at 11,600 *x* g for 10 minutes in a MiniSpin® plus centrifuge. After the centrifugation step, the supernatant was analyzed for DCF left in the liquid.

The adsorption capacity at time t (in µg g^{-1}) was calculated using Equation 1:

$$q_t = V(C_0 - C_t)/w \quad \text{----------} \quad (1)$$

Percentage of DCF removal at time,'t' was calculated using Equation 2:

$$\%\, removal\ (\%R) = \left\{\left(\frac{C_0 - C_e}{C_0}\right) 100\right\} \text{----------} \quad (2)$$

Where, C_0, C_t, and C_e represent the concentrations of DCF (mg L^{-1}) at the beginning of the experiment, at time t (min), and at equilibrium, respectively; V is the volume of the solution used for the study (L); and *w* is the mass of biochar used (g).

2.2.4 DCF Analysis

Analysis of DCF was performed by LDTD-MS/MS technique as described by Lonappan et. al (Lonappan et al., 2016a). The analytical set-up consisted of LDTD-APCI (Laser diode thermal desorption-atmospheric pressure chemical ionization) source (LDTD T-960, Phytronix Technologies, Quebec, Canada) mounted on a TSQ Quantum access triple quadruple mass spectrometer (Thermo Scientific, Mississauga, Ontario, Canada). Diclofenac-d_4 was used as the internal standard and quantification of DCF was carried out with the help of a calibration curve (1-1000 µg L^{-1}).

2.2.5 Statistical analysis

All of the adsorption related experiments were carried out in duplicates. Data presented are the mean values with standard deviation (±SD). Statistical significance of the measured values was analyzed using one-way ANOVA and analysis carried out with 95% confidence level using Sigmaplot, version 11.0 (Systat Software Inc., Germany) and the results which had $p < 0.05$ were considered as significant.

3. Results and discussion

3.1 Characterization of non-functionalized and functionalized biochars

Particle size distribution of biochars as obtained and micro-biochars are given in Fig.1. The mean particle size of the particles was recorded to be ≈36.3 µm, ≈30.6 µm, and ≈ 32.6 µm, respectively for BC-PW, BC-PM AND BC-AS. Particle size usually plays important role in properties, such as surface area, zeta potential etc. Moreover, according to IUPAC (The International Union for Pure and Applied Chemistry), particles having less than 100 µm, are microparticles (Lonappan et al., 2016b; Vert et al., 2012). In this study, the particles used were clearly microparticles. Moreover, after organic acid treatment, the samples were also subjected to particles size measurements. After organic acid treatment and consecutive drying, the micro-biochars were mostly agglomerated (data not shown). All the micro-biochars were having pH values ranging from 8.5-10 and mixing with organic acids might have changed the surface charges. This can be due to the agglomeration of micro-biochars after functionalization.

Figure 2 presents the scanning electron micrographs of micro-biochar and representative images from biochar functionalized using citric acid. For pinewood biochar, the surface texture remained the same; however, a more porous structure was observed. Previously, similar results were observed for activation using phosphoric acid (Shamsuddin et al., 2016). Moreover, in comparison with raw micro-biochar, the porous structures were more visible after acid treatment for all the biochars under study and this could be due to the removal of impurities by acid washing (Gai et al., 2014). However, the BC-PM biochar particles became clustered after citric acid treatment as observed through micrographs. Further, this observation can be attributed to the change in zeta potential (Table 3). Zeta potential changed from -39.8 mV to 3.1 mV after citric acid treatment. This change in surface charges might have affected the charge distribution and density resulting in agglomeration of micro-biochar.

3.2 Functionalization effect on different Biochars

Figures 3, 4 and 5 present the FTIR spectra of functionalized pine wood, pig manure, and almond shell biochars, respectively using various acids by different methods. An FTIR image on the left-hand side represents the results of functionalization by autoclaving whereas FTIR images on the right-hand side show functionalization effects by shaking method. PW, PM and AS represents the raw untreated pinewood, pig manure, and almond shell biochars and the number represents the molarity of the acid used. In each notation, the first letter stands for the acid; A- acetic acid, C- citric acid, M- maleic acid, O- oxalic acids and T- tartaric acid. The second letter in each notation stands for autoclave or shaking method, respectively by A and S. Correlation between FTIR results, effect of organic acids and changes in biochars surfaces has been explained in detail in the sections following this section.

Table 2 presents the changes in the elemental composition of biochars before and after treatment with various organic acids. In Table 3, results related to zeta potential, total acidic functional groups and percent changes in FTIR peaks of biochars before and after citric acid treatment have been tabulated.

3.2.1 Pinewood biochar

Previous studies reported similar FTIR profile as shown in Fig. 3 for the biochars derived from pine wood (Domingues et al., 2017). Pinewood derived biochars are usually rich in –OH groups (at 3400 cm^{-1}) from H_2O as well as from phenolic components), aliphatic functional groups and -CO (oxygenated functional groups of cellulose, hemicellulose, and methoxy groups of lignin) (Bruun et al., 2011; Domingues et al., 2017; Pradhan and Sandle, 1999). Treating pinewood biochar with acetic acid at high temperatures (autoclaving) did not lead to significant changes in the biochar surface chemistry. However, with simple shaking, a broad and strong peak was observed around 3300 cm^{-1} and which can be attributed to the O-H stretching possibly due to the formation of carboxylic acid groups (George et al., 1971; Max and Chapados, 2004; Ojamäe et al., 2006).

Furthermore, a noticeable increase in peak intensity was observed after shaking with acetic acid and which could be due to the increase in -COOH groups (Table 3). About 30% difference in peak intensity was observed and which can be directly correlated to approximately 30% increase in -COOH groups. Previous studies have also demonstrated the ability of oxidizing agents, such as acids on the introduction of surface oxygen complexes on carbons(Pradhan and Sandle, 1999). However, concentration (molarity) effect was minimal for acetic acid (approximately 30 % difference in peak intensities with all molarities) for the functionalization of BC-PW. A similar observation was made for citric acid as well. Instead of autoclaving, simple shaking resulted in a better result with citric acid. With citric acid and under shaking conditions, about 40% difference in peak intensity was observed and which can be directly correlated to approximately 40% increase in -COOH groups. Similarly, previous studies also demonstrated the semi-quantification of functional groups using FTIR (Haberhauer et al., 1998; He et al., 2018). Thus, organic acid treatment/functionalization required extended contact periods than intense reaction conditions, such as high temperature. Activation/mineral acid treatments are usually being carried out at very high temperatures and for a very short period (Pradhan and Sandle, 1999; Shamsuddin et al., 2016; Tan et al., 2015).The molarity of citric acid used had a positive impact on the grafting of COOH groups on the surface of BC-PW. With high concentrations of citric acid, an increase in the intensity of the COOH peaks was observed. For malic acid also, simple shaking resulted in better peak intensities. However, specific COOH functional groups on biochar surface were not identified with malic acid treatment. The same behavior as the malic acid was observed with oxalic acid and tartaric acid for BC-PW. Variation in elemental composition was observed in almost all samples. In most of the cases, differences in “O” content can be attributed to the formation of COOH group on biochar surface. Previous studies also observed introduction of surface oxygen complexes with acid treatment (Pradhan and Sandle, 1999; Shamsuddin et al., 2016). Moreover, as described in Table 3, considerable changes in properties, such as zeta potential and quantity of total acid functional groups further provided insight into the quantifiable effects of functionalization(Rouissi et al., 2011). For instance, 23.6% increase in total acidic functional groups was observed after citric acid functionalization. It has been reported that,

similar changes can significantly improve the biochar properties for several applications ranging from environmental remediation to energy storage and catalytic applications (Liu et al., 2015).

3.2.2 Pig manure biochar

A mixture of complex functional groups mainly composed of mineral and organic matter along with the presence of heavy metals can be observed in biochar derived from pig manure (Lonappan et al., 2017; Tsai et al., 2012). The similar observation was made with FTIR spectrum of BC-PM in this study. From Fig.4, it can be observed that autoclaving or shaking with acetic acid did not make a big difference in the biochar surface chemistry in comparison with the untreated biochar. However, with citric acid, changes in peak intensities were observed even though the formation of new peaks or peaks corresponding to COOH groups was not observed. The same trend was observed with malic acid as well no significant change in surface chemistry was observed. From FTIR spectra, oxalic acid was found to be effective for the functionalization of pig manure biochar. Peaks in FTIR spectrum became much more visible and peak intensities increased while treating with oxalic acid. Between autoclaving and shaking, shaking method was found to be effective for the functionalization. Moreover, while shaking, a high concentration of oxalic acid turned out to be better. This was further confirmed by the elemental composition (Table 2). About 42 %, oxygen was present in the initial raw pig manure biochar and, which was decreased with most of the functionalization methods. This factor points out the possibility of the addition of COOH groups on biochar. Nevertheless, with oxalic acid, nearly 40 % oxygen was observed after functionalization which points towards the possibility of preservation/addition of further COOH groups on biochar surface. On the other hand, functionalization using tartaric acid did not lead to any changes in surface functional groups.. The previous study on adsorption of carbaryl and atrazine on pig manure-derived biochars proved a significant increase in surface area and pore volume after treatment with HCl (Zhang et al., 2013).In addition, comparing with other two biochars, organic acids had minimal functionalization effect on pig manure biochar. As tabulated in Table 4, only about 10% increase in total acid functional groups was observed after citric acid functionalization with pig manure biochar whereas,

with other two biochars, it was more than 20%. These results were further confirmed by the minimal to no change in peak intensities in FTIR spectra (Fig.4).

3.2.3 Almond shell biochar

From Fig.5, it is evident that neither autoclaving nor shaking with acetic acid result in a huge difference in the biochar surface chemistry in comparison with the untreated biochar. However, while shaking with citric acid, a broad and strong peak was observed around 3300 cm^{-1} and which can be attributed towards the O-H stretch due to the formation of carboxylic acid groups. Furthermore, the intensity of the peaks increased after shaking with acetic acid and which could be due to the increase in COOH group. Instead of autoclaving, simple shaking gave better result with citric acid. Approximately 45% increase in peak intensity was observed under shaking conditions with 0.5 M citric acid. On performing semi-quantification with FTIR data, approximately 45% increase in –COOH groups was observed. (Haberhauer et al., 1998; He et al., 2018) Moreover, the molarity of citric acid used had a negative impact on the grafting of COOH groups on the surface of pine wood biochar. A 0.5 M citric acid was found to effective for optimal functionalization from peak intensities in FTIR spectrum. For malic acid and oxalic acid also, simple shaking resulted in better peak intensities. However, specific COOH functional groups on biochar surface were not identified with malic and oxalic acid treatments. Also, tartaric acid was not effective agent for functionalization: peak intensities were almost similar in comparison with untreated biochar. However, a peak around 2400 cm^{-1} was observed and which was attributed towards the presence of aldehyde groups. From Table 2, small variations in elemental composition were observed in almost all samples. However, these very small differences cannot be attributed to the presence of any surface functional groups. For most of the cases, differences in “O” content can be due to the formation of COOH groups on the biochar surface. Moreover, 26.2% increase in total acidic functional groups were observed after citric acid functionalization (2M; under shaking conditions) and which further confirmed the quantifiable effects of functionalization.

3.3 Diclofenac removal by functionalized biochars

It has been observed that 2M citric acid was found to be effective for grafting COOH functional groups on pine wood and almond shell biochar surface. Thus, biochars treated with citric acid were tested for its removal efficiency of diclofenac as a model emerging contaminant as demonstrated in Fig. 6. From previous studies (Lonappan et al., 2017), it was observed that the equilibrium was obtained in 5 hours and hence the adsorption was carried out for 5 hours.

Pig manure biochar was very effective (approx. 100% removal) for the removal of DCF under similar conditions(Lonappan et al., 2017). Thus, further improvement in removal efficiency was not observed after functionalization. With citric acid functionalization, adsorption capacities of pine wood and almond shell biochars were significantly ($p < 0.05$) increased at both concentrations under study. For pinewood biochar, at 500 µg L^{-1} DCF, the removal efficiency increased from 65 % to 80 %. For almond shell biochar and under similar conditions, removal efficiency increased from 58 % to 84 %. The increased removal efficiency can be attributed to the induction/ increased number of COOH groups on the biochar surface along with the possible increased surface area. It has been reported that COOH groups are effective for the removal of metal ions from soil (Uchimiya et al., 2012). Several interactions, such as p-p electron donor-acceptor interactions, hydrophobic interactions, hydrogen bonding and Van der Waal forces are involved in adsorption (Jung et al., 2015; Nam et al., 2015). Here, the presence of possible COOH groups might enhance these interactions and thus the adsorption capacity of biochars has been increased. Zeta potential measurements before and after functionalization have been given as Table 3. An increase in zeta potential was observed for citric acid treated biochar. The changes in zeta potential can be directly correlated with observed FTIR results of approximately 40-50% increase in -COOH functional groups with BC-PW and BC-AS. Moreover, Zp changes were considerably higher (Table 4), and certainly had a positive effect on the adsorption and removal of DCF. Acidic functional groups can enhance DCF adsorption (Lonappan et al., 2017) and after citric acid functionalization, more than 10% increase in total acidic functional groups were observed with all biochars (Table 4). This 10%

increase in functional groups has led to significant 25-40 % ($p < 0.05$) improvement in removal efficiency with all micro-biochars used for the study.

Biochar is gaining a much wider attention now a days as a sustainable platform carbon material (Liu et al., 2015). The application of organic acids for the functionalization of biochars is an economically viable and environmentally friendly method and that will undeniably help to create a sustainable future. Eventhough this study demonstrated one potential application of diclofenac removal through functionalized biochar; this does not limit the application of organic acid functionalized biochar in a single domain. These functionalized biochars can find applications in catalysis, energy storage, environmental remediation and agriculture. Moreover, this study reported variable behavior of organic acids with different biochars (in terms of method of production and feedstock selection). This further diversifies the surface changes or functional groups on biochar surface and opens up door for applications.

4. Conclusions

Biochar produced from the feedstock, such as pine wood, pig manure, and almond shell were functionalized using acetic acid (CH_3COOH), citric acid ($C_6H_8O_7$), tartaric acid ($C_4H_6O_6$), oxalic acid ($C_2H_2O_4$), malic acid ($C_4H_6O_5$). Two functionalization methods, simple shaking overnight at room temperature and autoclaving were tested and compared for the effectiveness of functionalization. For pinewood biochar, citric and acetic acid was found to be effective and citric acid being more efficient (approximately 40 % increment in –COOH groups on the surface). Tartaric and oxalic acids were found to be effective for pig manure biochar, however, the effect of both was minimal on functionalization. Citric and oxalic acids were found to be effective for almond shell biochar and citric acid being more efficient (approximately 45 % increment in –COOH groups on the surface). Biochar composition (feedstock) had a significant effect while reacting with the organic acids. Biochar surfaces responded differently with all acids. Moreover, the concentration (molarity) of acids had little to no effects on extent of functionalization. Simple shaking over a long period was found to effective

over autoclaving at high temperature for short period. Overall, citric acid was found to be the efficient organic acid for effective functionalization (COOH) on biochar surface. Citric acid functionalization increased total acidic functional groups by 23.6 %, 10.2 %, and 26.2 % for pine wood, pig manure and almond shell, respectively. Citric acid functionalized biochars gave significant removal ($p < 0.05$) of DCF at equilibrium.

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Table 1: Molarities of various acids used for functionalization of biochars

Acetic acid	Citric acid	Tartaric acid	Oxalic acid	Malic acid
2 M	2 M	1M	0.1M	2 M
1M	1M	0.5M	0.5M	1M
0.5M	0.5	0.1M	1M	0.5M

Table 2: Elemental composition of biochars - before and after functionalization

Acid and method used	% C (±10%)			% H (±10%)			% N (±10%)			% S (±10%)			% O (approximately)		
	BC-PW	BC-PM	BC-AS	BC-PW	BC-PM	BC-AS	BC-PW	BC-PM	BC-AS	BC-PW	BC-PM	BC-AS	BC-PW	BC-PM	BC-AS
Pine wood biochar	78	-	-	3.1	-	-	0.3	-	-	< 0.5	-	-	18.1	-	-
Pig manure biochar	-	52.5	-	-	1.4	-	-	3.0	-	-	< 0.5	-	-	42.61	-
Almond shell biochar	-	-	66.6	-	-	2.7	-	-	1.0	-	-	< 0.5	-	-	29.13
0.5M Acetic acid - autoclave	70.3	61.1	74.5	3	1.8	2.3	0.4	3.5	1.4	< 0.5	< 0.5	< 0.5	25.8	33.09	21.32
1 M Acetic acid- autoclave	68.4	60.9	69.1	3	2.2	3.0	0.4	3.8	1.1	< 0.5	< 0.5	< 0.5	27.7	32.63	26.30
2 M Acetic acid- autoclave	77.3	69.7	50.6	3.1	2.3	2.2	0.3	4.1	1.7	< 0.5	< 0.5	< 0.5	18.8	23.48	45.00
0.5 M Acetic acid- shaking	74.7	64.2	74.1	3	1.3	2.9	0.5	3.7	1.2	< 0.5	< 0.5	< 0.5	21.3	30.25	21.37
1M Acetic acid- shaking	77.5	61.9	75.3	3.4	1.4	2.9	0.3	3.8	1.1	< 0.5	< 0.5	< 0.5	18.3	32.33	20.21
2M Acetic acid- shaking	76.1	63.9	72.8	3.5	1.9	2.8	0.2	3.7	1.1	< 0.5	< 0.5	< 0.5	19.7	29.96	22.84
0.5M Citric acid - autoclave	77.7	69.5	70.0	3.3	2.2	2.8	0.3	3.9	1.2	< 0.5	< 0.5	< 0.5	18.2	23.90	25.58
1M Citric acid - autoclave	96.4	65.8	70.5	4.1	2.4	2.7	0.6	4.4	1.3	< 0.5	< 0.5	< 0.5	ND	26.90	25.01
2M Citric acid- autoclave	62.3	69.3	69.6	3.5	2.3	2.7	0.2	4.0	1.3	< 0.5	< 0.5	< 0.5	33.5	23.95	25.84
0.5M Citric acid- shaking	76.3	66.5	72.7	3.1	1.5	3.1	0.5	4.1	1.1	< 0.5	< 0.5	< 0.5	19.6	27.47	22.60
1M Citric acid- shaking	79.7	65.5	73.3	3.2	1.7	3.0	0.3	3.9	1.2	< 0.5	< 0.5	< 0.5	16.3	28.38	22.08

2M Citric acid- shaking	70.9	64.6	70.1	3.3	1.8	3.0	0.3	3.8	1.1	< 0.5	< 0.5	< 0.5	25	29.26	25.29
0.1M Tartaric acid- autoclave	78.6	53.5	72.4	3.2	2.7	3.1	0.3	2.9	1.1	< 0.5	< 0.5	< 0.5	17.4	40.44	22.98
0.5M Tartaric acid- autoclave	80	53.8	74.7	3.1	2.5	3.2	0.3	2.9	1.2	< 0.5	< 0.5	< 0.5	16.1	40.20	20.45
1M Tartaric acid- autoclave	75.5	56.3	72.8	2.8	2.6	2.9	0.5	2.9	1.1	< 0.5	< 0.5	< 0.5	20.7	37.64	22.77
0.1M Tartaric acid- shaking	78	54.8	72.0	3.4	2.4	2.8	0.3	3.1	1.3	< 0.5	< 0.5	< 0.5	17.8	39.22	23.37
0.5M Tartaric acid- shaking	50.2	78.9	68.8	2.9	3.3	2.9	2.7	0.4	1.0	< 0.5	< 0.5	< 0.5	43.7	16.86	26.76
1M Tartaric acid- shaking	80.1	56.5	70.1	3.4	2.4	2.7	0.3	3.2	1.2	< 0.5	< 0.5	< 0.5	15.7	37.39	25.46
0.5M Malic acid- autoclave	75.2	63.6	73.9	3.1	1.9	2.8	0.5	4.1	1.2	< 0.5	< 0.5	< 0.5	20.7	29.91	21.61
1M Malic acid- autoclave	76	67.6	75.2	3.3	2.1	2.8	0.5	3.9	1.3	< 0.5	< 0.5	< 0.5	19.7	25.90	20.21
2M Malic acid- autoclave	77.3	63.5	69.3	3.4	2.2	3.1	0.5	3.7	1.2	< 0.5	< 0.5	< 0.5	18.3	30.02	25.92
0.5M Malic acid- shaking	78.9	66.8	66.3	3.1	2.1	2.9	1	3.8	1.6	< 0.5	< 0.5	< 0.5	16.5	26.80	28.71
1M Malic acid- shaking	79.4	62.2	73.7	3	1.8	3.2	0.3	4.0	1.1	< 0.5	< 0.5	< 0.5	16.8	31.53	21.54
2M Malic acid- shaking	76.7	62.6	73.9	3	2.1	2.8	0.3	3.8	1.2	< 0.5	< 0.5	< 0.5	19.5	31.03	21.68
0.1M Oxalic acid- autoclave	73.3	53.6	73.6	3	1.8	2.6	0.4	3.1	1.1	< 0.5	< 0.5	< 0.5	22.8	40.98	22.16
0.5M Oxalic acid- autoclave	58.5	56.8	72.9	2	1.9	2.7	3.2	3.2	1.3	< 0.5	< 0.5	< 0.5	35.8	37.63	22.60
1M Oxalic acid- autoclave	83.3	57.6	70.0	3.3	1.7	2.9	0.2	3.1	1.0	< 0.5	< 0.5	< 0.5	12.7	37.06	25.64
0.1M Oxalic acid- shaking	76.9	54.7	71.3	3.2	1.9	2.4	0.2	3.0	1.1	< 0.5	< 0.5	< 0.5	19.2	39.89	24.67
0.5M Oxalic acid- shaking	77.7	57.2	72.6	3.2	1.6	2.2	0.4	3.2	1.2	< 0.5	< 0.5	< 0.5	18.2	37.49	23.44
1M Oxalic acid- shaking	79.3	75.1	76.1	3.3	3.0	2.7	0.3	0.6	1.1	< 0.5	< 0.5	< 0.5	16.6	20.85	19.56

Table 3: Total acidic functional groups, zeta potential and shift in FTIR peaks in functionalized and non-functionalized biochars

Biochar	Acidic functional groups ($mmolg^{-1}$)		Transmittance from FTIR (%)		Zeta potential (mV)	
	Raw micro biochar	After citric acid functionalization	Raw micro biochar	After citric acid functionalization	Raw micro biochar	After citric acid functionalization
Pine wood	2.9	3.8	75.6	36.1	-55.3	-0.6
Pig manure	3.5	3.9	71.3	61.1	-39.8	3.1
Almond shell	3.1	4.2	67.6	28.3	-48.7	1.2

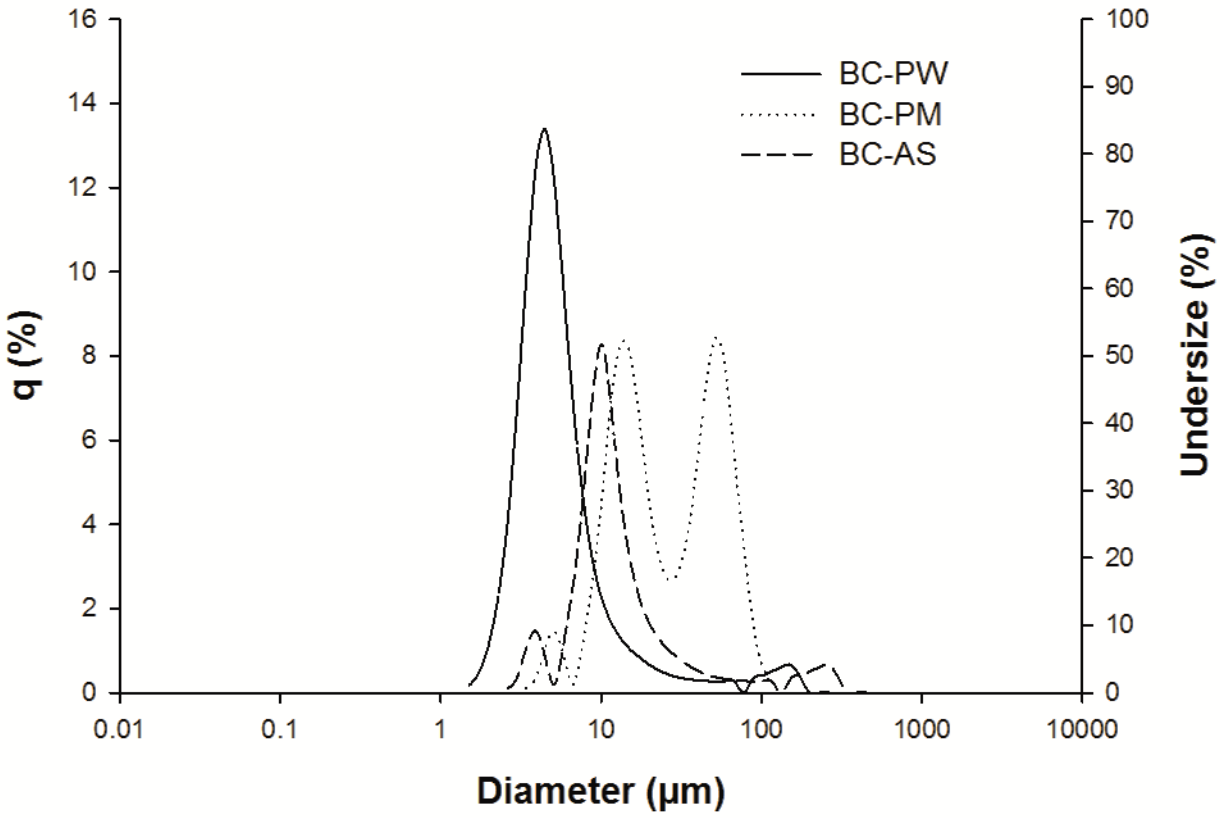

Figure. 1: Particle size of biochars used

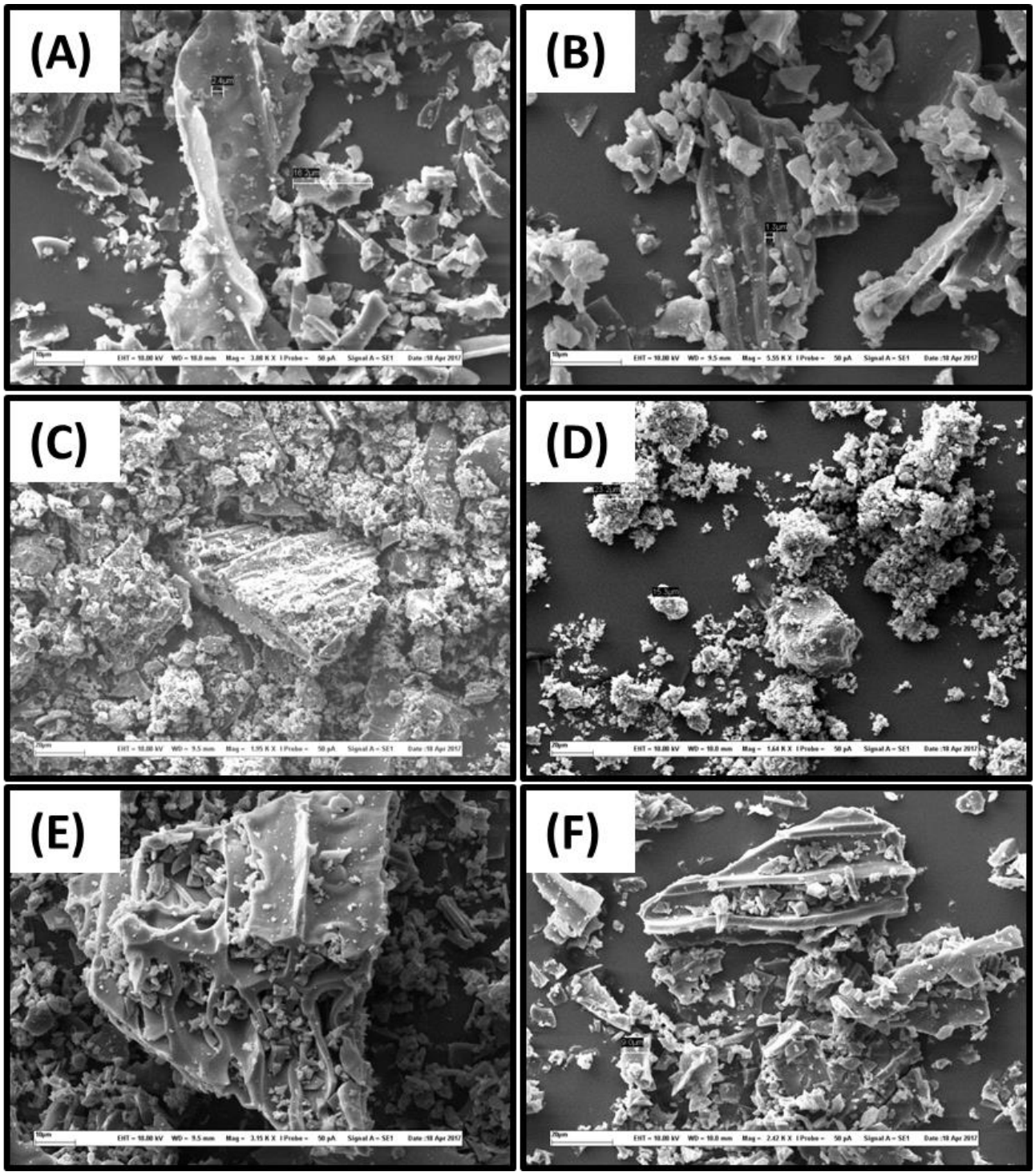

Figure 2: Representative scanning electron micrographs of non-functionalized and citric acid functionalized biochar. A- raw pine wood biochar; B-functionalized pine wood biochar; A- raw pine wood biochar; B-functionalized pine wood biochar; C- raw pig manure biochar; D-functionalized pig manure biochar; E- raw almond shell biochar; F-functionalized almond shell biochar

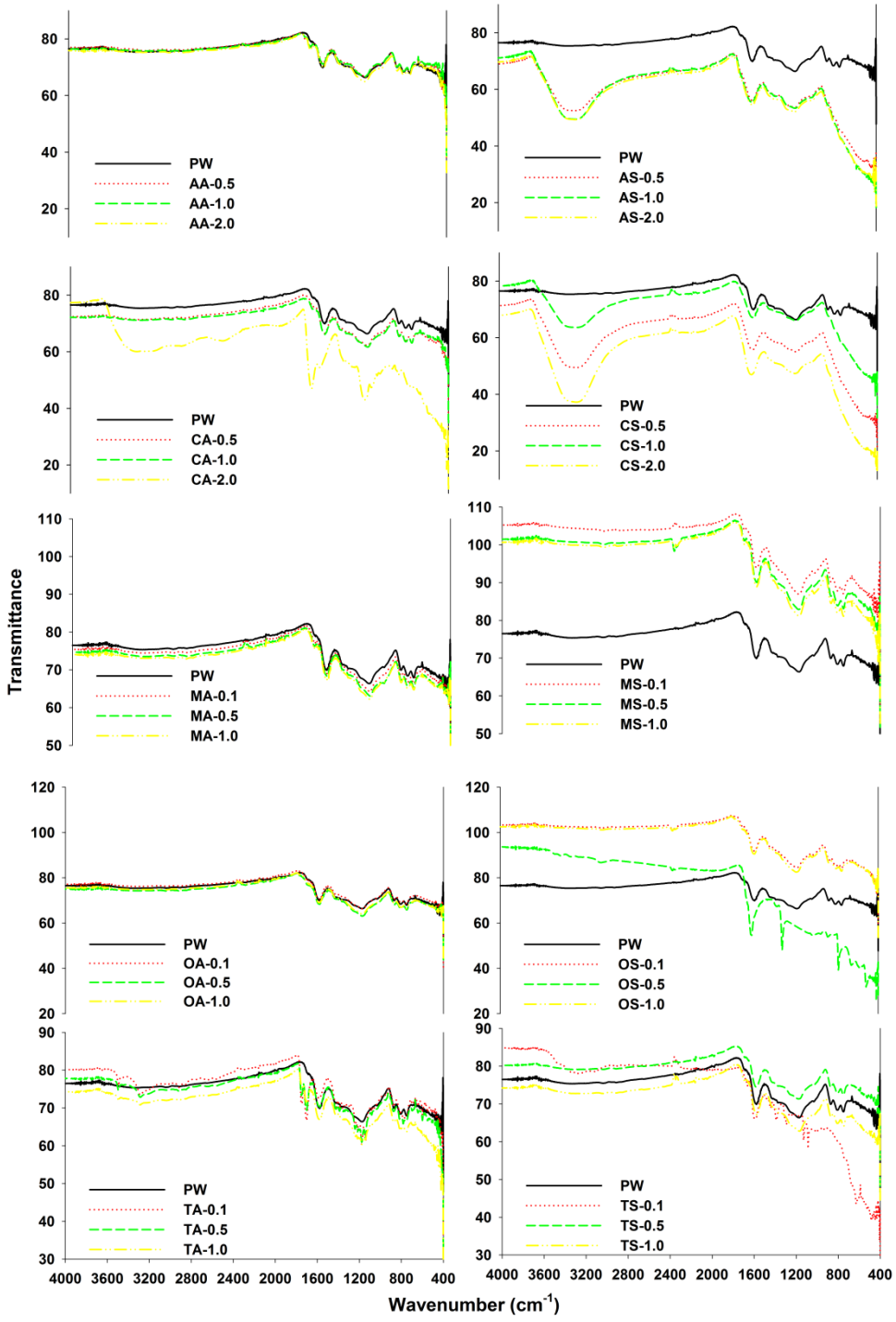

Figure 3: FTIR spectrum – functionalization of pinewood biochar using organic acids

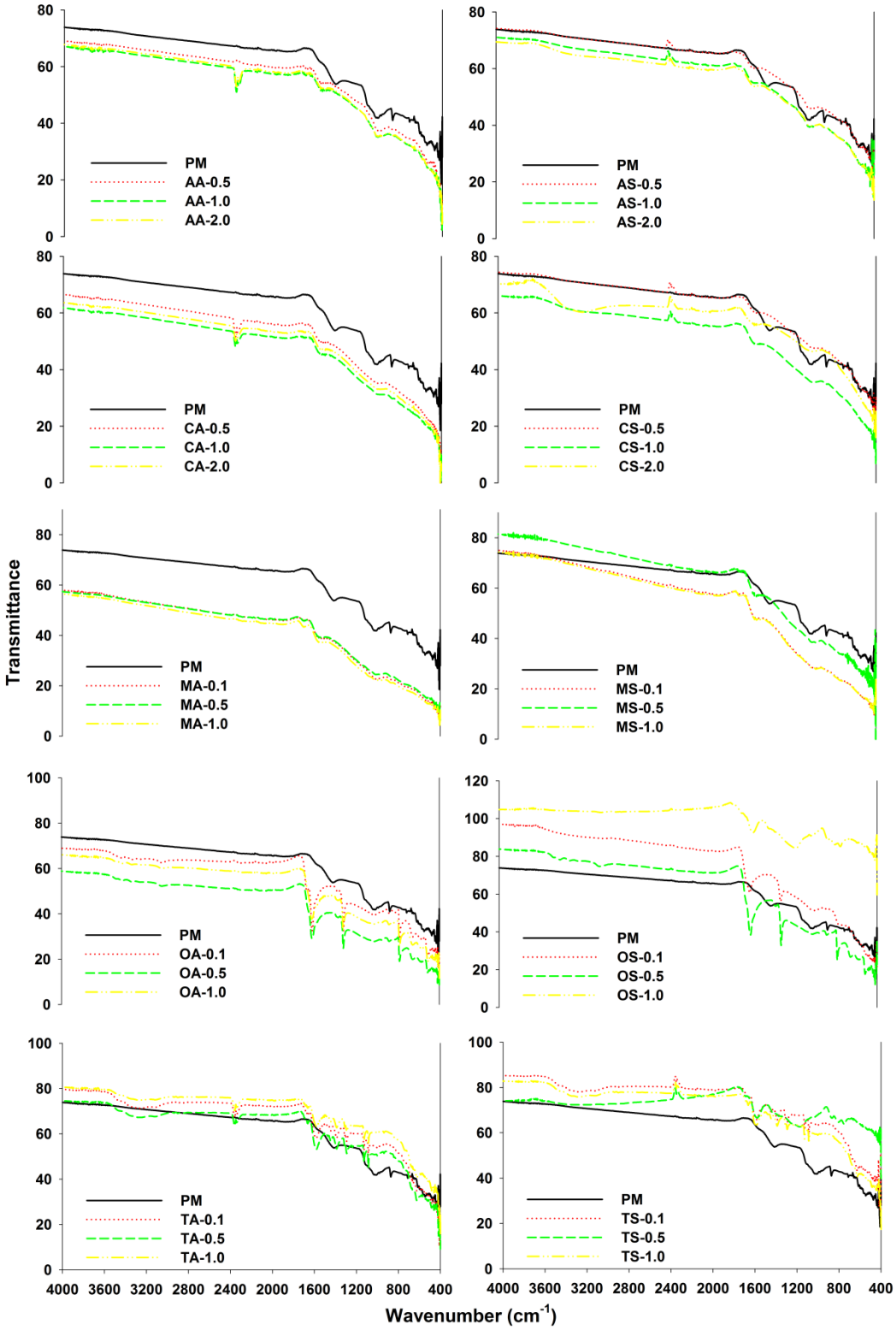

Figure 4: FTIR spectrum – functionalization of pig manure biochar using organic acids

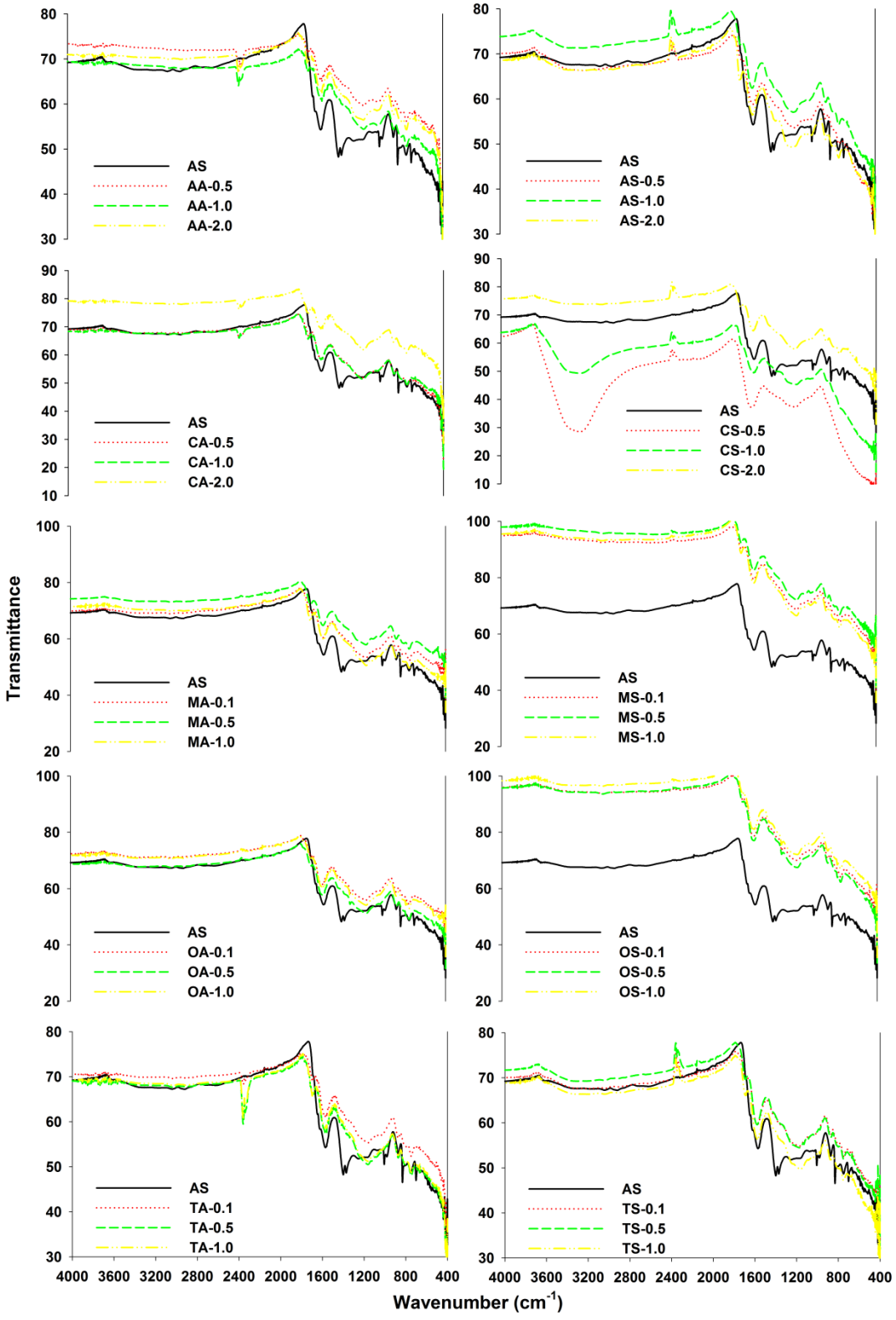

Figure 5: FTIR spectrum – functionalization of almond shell biochar using organic acids.

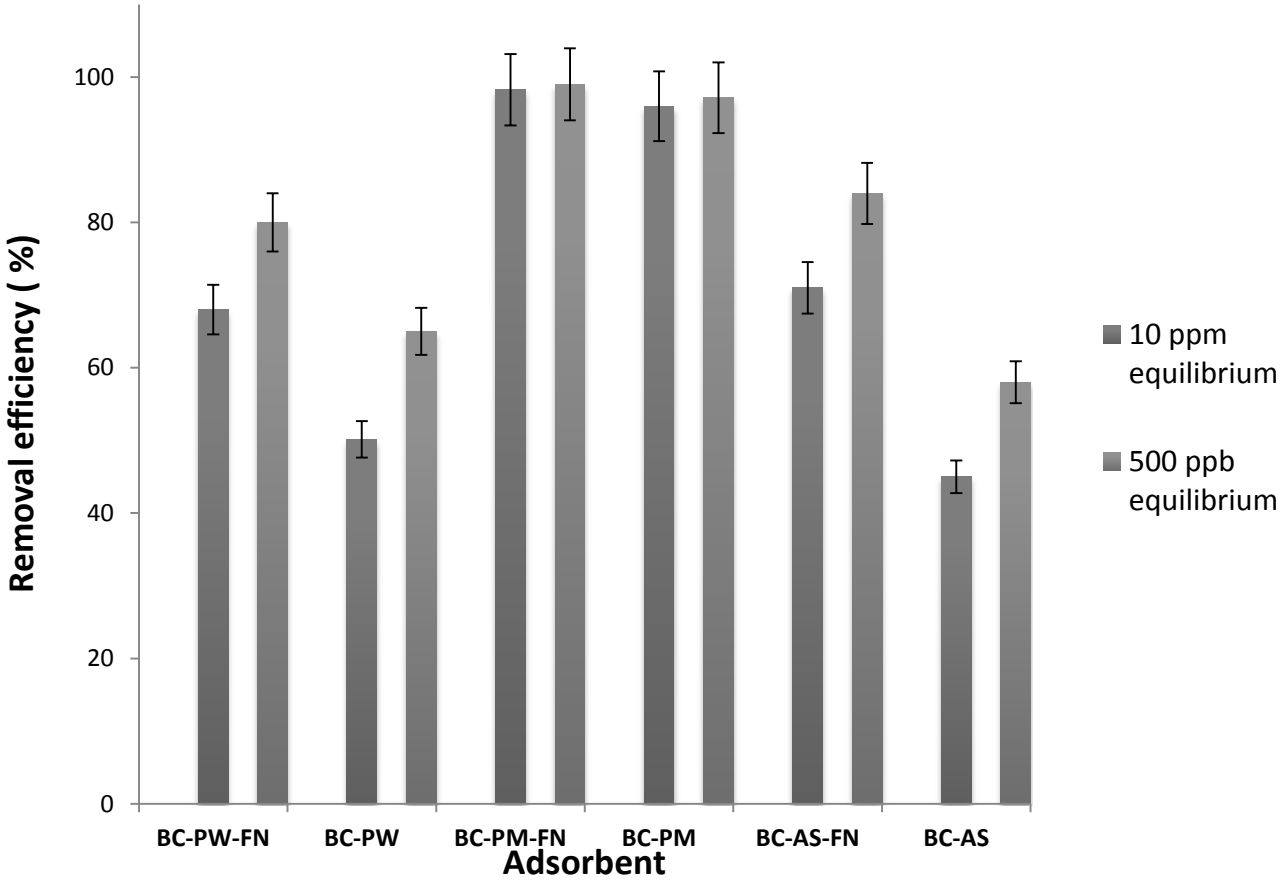

Figure 6: Functionalized biochars and removal of diclofenac

CHAPTER 4

LACCASE ENZYMES: PRODUCTION; IMMOBILIZATION OF ENZYMES ON MICRO-BIOCHARS AND DEGRADATION OF DICLOFENAC

PART 1

AGRO-INDUSTRIAL PRODUCED LACCASE FOR DEGRADATION OF DICLOFENAC AND IDENTIFICATION OF TRANSFORMATION PRODUCTS

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RÉSUMÉ

Le diclofenac, un anti-inflammatoire répandu, est récalcitrant dans divers milieux aquatiques et représente une menace pour plusieurs espèces aquatiques et terrestres. La dégradation enzymatique des contaminants émergents, souvent les micropolluants, a gagné de l'intérêt au cours des dernières années. Cependant, les coûts de production d'enzymes demeurent élevés. Dans cette étude, la laccase, une enzyme ligninolytique, a été produite par des champignons de pourriture blanche *Tremetes versicolor (ATCC 20869)* en utilisant les résidus agro-industriels suivants : le marc de pomme (AP), les résidus solides de pâte et papier (PPSW) et les fibres alfa comme substrat. Plusieurs inducteurs de production de laccase tels que le tween 80 (0.1% (w/w)), l'alcool veratryle (3 mM Kg^{-1}), CuSO4 (3 mM Kg^{-1}) et le Rouge de Phénol (3 mM Kg^{-1}), ont été utilisés pour améliorer la production de laccase. Des activités de laccase maximum de 49.16 ± 4.5 U/gds (units/gram dry substrate), 52.4 ± 2.2 U/gds and 14.26 ± 0.8 U/gds ont été obtenues dans des conditions expérimentales optimales à partir de AP, PPSW et de fibre alfa, respectivement. Ensuite, la cinétique de dégradation du DCF par la laccase a été étudiée. À des concentrations représentatives de celles retrouvées dans l'environnement, un pH de 4.5 et une température de 50°C se sont avérés être les conditions optimales pour la dégradation du DCF par la laccase. Le 3′-hydroxydiclofenac, le 4′-hydroxydiclofenac and le 5-hydroxydiclofenac ont été identifiés comme étant les principaux produits de transformation pendant les premières 5 heures de dégradation. Cependant, après 24 heures de dégradation, ni le DCF, ni aucuns autres produits de transformation ont été identifiés. Ainsi, le mécanisme de dégradation proposé implique une hydroxylation suivi par une ouverture des cycles et une minéralisation finale en CO_2, NH_3, H_2O et HCl.

Mots clés : Diclofenac; Laccase; Dégradation; Produit de transformation; Marc de pomme; Cinétique, fibre alfa; résidus solides de pâte et papier

ABSTRACT

A widely used anti-inflammatory drug, diclofenac (DCF) is recalcitrant in many environmental compartments and poses threat to several aquatic and terrestrial organisms. Enzymatic degradation of emerging contaminants which are often micropollutants, has gained interest for the past few years. However, production of enzymes often incurs high costs. In this study, ligninolytic enzyme laccase was produced by white rot fungi *Tremetes versicolor (ATCC 20869)* using agro-industrial residues, apple pomace (AP), Pulp and paper solid waste (PPSW) and alfa fibers as substrates. Various known inducers for laccase production, such as tween 80 (0.1% (w / w)), veratryl alcohol (3 mM Kg^{-1}), CuSO4 (3 mM Kg^{-1}) and phenol red (3 mM Kg^{-1}) were used to enhance laccase production. A maximum laccase activity of 49.16± 4.5 U/gds (units/gram dry substrate),52.4±2.2 U/gds and 14.26±0.8 U/gds was obtained from apple pomace, PPSW and alfa plant fibers, respectively at optimal experimental conditions. Further, the kinetics of the laccase mediated degradation of DCF was studied. At environmentally relevant concentration of DCF (500 µg L^{-1}), laccase catalyzed degradation followed first order kinetics. At environmentally relevant concentrations pH of 4.5 and temperature of 50 °C was found to be optimal for the effective degradation of DCF with laccase. 3′-hydroxydiclofenac, 4′-hydroxydiclofenac and 5-hydroxydiclofenac were identified as the major transformation products during the initial five hours of degradation. However, after 24 hours of degradation, neither DCF nor any transformation products were identified so that the proposed degradation mechanism involved hydroxylation followed by ring opening and final mineralization to CO_2, NH_3, and H_2O and HCl.

Keywords: Diclofenac; Laccase; Degradation; Transformation products; Apple pomace; Kinetics; Alfa fibers; Pulp and paper solid waste

1. Introduction

The use of pharmaceuticals is vital for the prevention, treatment, and maintenance of productivity in human as well as other organisms [1]. Besides its inevitable applications, these pharmaceuticals spawn concerns over environmental pollution due to their frequent occurrences in wastewater as well as drinking water [2]. After consumption, excess, partially metabolized and conjugate forms usually end up in wastewater treatment plant. These emerging contaminants (ECs) are often persistent and induce unwanted biological effects in many aquatic as well as terrestrial organisms even at very low or micro concentrations. Diclofenac (DCF) is a non-steroidal anti-inflammatory drug (NSAID) and its global human consumption is estimated to be more than 1443 ± 58 tons [3]. Owing to its huge consumption and poor degradation in wastewater treatment plants [4], residues of DCF and its metabolites are frequently detected in wastewater [5], surface water [6] and even in drinking water [5].The adverse effects and toxicity of DCF is well established for several aquatic organisms and microbial communities [2, 4-5] in fragile river ecosystems. Furthermore, the mixed toxic concerns over the untreated DCF along with its own metabolites, other ECs, organic and inorganic contaminants is still undeciphered [2]. Thus, DCF need to be treated at its primary sink and the transformation products must be properly identified and tested for any unwanted potential toxic effects. Most of the currently existing methods are energy and cost-intensive and the quest for new, alternative sustainable and eco-friendly treatment methods must be initiated.

Ligninolytic enzymes are known for detoxification/degradation of recalcitrant pollutants [7-8]. Amongst ligninolytic enzymes, laccases (1.10.3.2, p-diphenol: dioxygen oxidoreductases) have been widely recognized for biotechnological and waste treatment applications due to simple requirements for laccase catalysis (presence of substrate and O_2), as well as its extra stability and minor inhibition possibilities in comparison with other enzymes of the same class[9]. A wide variety of microorganisms has been used for the production of laccases, of which different fungal species are well known due to their salient capabilities for enhanced production. For fungal cultures, various fermentation techniques, such as submerged fermentation, solid-state fermentation and semi-solid-state

fermentation were utilized and a wide variety of substrates has been used as the fermentation media [8]. Moreover, the application of various inducers enhances the laccase production [10]. High production costs are often associated with enzyme production, particularly in large-scale production, and which is usually associated with the expensive medium for the microbial growth. Thus, investigation on the utilization of wastes from agriculture and food processing industries as substrates is getting much wider attention [8, 11]. However, selecting a suitable lignocellulosic material (substrate) having an appropriate Carbon/Nitrogen (C/N) ratio, micro-macro nutrients composition with the absence of potential inhibitors is important for the cost-effective production of laccase. In this study, juice industry residue, apple pomace (AP) has been used as one of the solid substrate for the production of laccase due to its vast availability in Canada. AP has been already proved as an excellent medium for the production of various enzymes and platform chemicals [12]. Moreover, life cycle analysis studies proved that the fermentation method to produce ligninolytic enzymes is environmentally sustainable for the disposal of the juice industry residuals [13]. Pulp and paper solid waste (PPSW) has been sleceted as another substrate due to its vast availability in Canada and rich lignocellulosic contents[14] . Moreover, proper disposal and management of PPSW is a major concern thorgh out the world[15-16]. Likewise, the feasibility for laccase production has been tested for the alfa plant fibers (otherwise known as needles grass - *Stipa tenacissima)* for the first time to the best of our knowledge. Needles grass is well known for its polyphenolic and lignin contents [17-18] and thus can be a suitable candidate for use as a substrate for laccase production. Hence, three agro-industrial residues, apple pomace dried alfa fibers and pulp and paper solid waste (PPSW) were used for the production of the ligninolytic enzyme, laccase. A well-known producer of laccase, *Trametes versicolor*- ATCC 20869 was selected as the suitable microorganism. Effect of various inducers, such as veratryl alcohol (3, 4-dimethoxybenzyl alcohol), Tween-80 ($C_{32}H_{60}O_{10}$), phenol red($C_{19}H_{14}O_5S$) and copper sulfate ($CuSO_4$) were studied for the production of laccase. Fermentation conditions were optimized in terms of substrate and inducer concentration. The supernatant suspension obtained from fermentation medium was concentrated using ultrafiltration. The crude enzyme suspension was used for the degradation of DCF at varying pH, temperatures,

enzyme activities and DCF concentrations and the optimum conditions for the degradation of DCF was determined. Moreover, kinetics of the laccase-mediated degradation of DCF was studied and transformation products were identified by tandem mass spectroscopy.

2. Materials and methods

2.1 Chemicals and reagents

The inducers, Tween 80, phenol red and copper sulfate pentahydrate were purchased from Fisher Scientific (Ottawa, ON, Canada). The 3,4-dimethoxybenzyl alcohol (veratryl alcohol) was purchased from Sigma- Aldrich, Canada. Reagents for buffer solutions (monosodium phosphate, disodium phosphate, and citric acid) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were purchased from Fisher Scientific (Ottawa, ON, Canada).

Diclofenac sodium salt (98%; CAS 15307-79-6), HPLC-grade methanol (MeOH, purity >99.8%), HPLC grade acetonitrile (ACN, assay 99.9%), glacial acetic acid (assay: ≥99.7% w/w) and ammonium hydroxide (NH4OH, 28–30% w/w) were purchased from Fisher Scientific (Ottawa, ON, Canada). The internal standard (IS), diclofenac-d_4 was obtained from C/D/N isotopes Inc. (Montreal, QC, Canada). Disodium ethylenediamine tetraacetic acid (Na_2H_2EDTA, 99%) was purchased from E-bay (Tokyo, Japan). Chromatography grade water was prepared in the laboratory using Milli-Q/Milli-RO Millipore system (Milford, MA, USA).

2.2 Production of laccase

2.2.1 Substrates

Juice industry residue, apple pomace was gifted by Vergers Paul Jodoin Inc, Quebec and used after drying overnight at 60 ±1 °C in a hot air oven to remove moisture. Pulp and paper solid waste (PPSW) was obtained from Kruger Wayagamack Inc. (Trois-Rivieres, Quebec, Canada) PPSW is a mixture of primary sludge (60%) and secondary sludge (40%). Alfa fibers were dried in a hot air oven and ground in Retsch RS 200' vibratory disc mill at 750 rpm for 10 minutes to obtain particles ranged

from 75-300 µm (ASTM 200 and 50 numbered sieves) which were used for the fermentation. Before fermentation substrates were adjusted to 75 ±1 % (w/w) moisture with sterile milli-Q water.

2.2.2 Microorganism

White-rot fungus, *Trametes versicolor* (ATCC 20869) was selected as a suitable organism for enzyme production due to its excellent potential. The culture was grown on potato-dextrose-agar (PDA) plates and incubated at 30 ± 1 °C for 8–10 days. The mycelial layer from the PDA plates was harvested under sterile conditions and used as the inoculum for fermentation. The culture plates were stored at 4 ± 1 °C and sub-cultured every 3 weeks.

2.2.3 Solid state fermentation

Experiments were carried out at 30 ± 1°C for 14 days in 500 mL Erlenmeyer flasks containing 20 g of the solid substrates (apple pomace, PPSW and alfa fibers). The solid medium containing 75 % *(w/w)* moisture was autoclaved at 121 °C for 20 minutes. The medium was inoculated with mycelium from 8-10 days old *Trametes versicolor* plate (diameter of petri dish = 90 mm) which was previously optimized to one plate for optimum growth and incubated at 30 ± 1°C for 14 days. In addition, inducers, such as tween 80 (0.1% (*w / w*; equivalent to 1g of tween 80 per Kg of dry substrate), veratryl alcohol (3 mM Kg^{-1} of dry substrate ; equivalent to 0.5 g of 3,4-dimethoxybenzyl alcohol (veratryl alcohol) per Kg of dry substrate), $CuSO_4$ (3 mM Kg^{-1} of dry substrate; equivalent to 0.75 g of copper sulfate pentahydrate per Kg of dry substrate), phenol red (3 mM Kg^{-1} of dry substrate; equivalent to 1.06 g of phenol red per Kg of dry substrate) and a mixture of all these inducers were used to enhance the production of laccase. The concentrations of the inducers were selected following previous studies [19]. Enzyme extraction and moisture adjustment (by adding sterile water, if needed) were carried out on alternative days starting from the 5^{th} day of fermentation, until 14^{th} day. Aeration was maintained by aerating the fermenting substrate under sterile conditions and by gentle mixing in alternative days.

2.2.4 Enzyme extraction

One gram of fermented sample was mixed with 9 ml of 50 mM sodium phosphate buffer of pH 6.5 (10/1:*v/w*). Vigorous agitation was carried out using a sterile metallic rod followed by vortexing for 10 minutes. The mixture was later centrifuged at 9000 *x* g for 30 min at 4° C. The supernatant collected was analyzed for enzyme activity.

2.2.5 Enzyme assay

Laccase activity was determined by measuring the rate of color generation due to the enzymatic oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to ABTS radical ion ($ABTS^{+}$) at 420 nm and pH 4.5. The extinction coefficient (ε) used was 36,000 M^{-1} cm^{-1} [20]. The assay solution contained 100 mM citrate phosphate as the buffer (pH 4.5), 0.5 mM ABTS and enzyme. Enzyme-ABTS mixture was incubated for four minutes and the average per minute increase in absorbance was taken. One unit of laccase activity (U L^{-1}) is defined as the production of 1 μM $ABTS^{+}$ per minute under assay conditions [21-22]

2.2.6 Enzyme concentration

Crude laccase obtained from apple pomace was concentrated by ultrafiltration using Sartorius stedim- SartoJet ultrafiltration system (Göttingen, Germany). Sartocon Slice Hydrosart Cassette- 10 kDa and 100 kDa were used as the filtration membranes. Harvested enzyme was filtered using 100 kDa membrane to remove the particles having size more than 100 kDa. Later, the filtrate was passed through 10 kDa and the retentate having particles of molecular weight less than 10 kDa have been discarded.

2.3 DCF degradation experiments - methodology

Degradation experiments were carried out under various experimental conditions, such as varying pH (from 2.5 to 12.5) and temperature (from 10°C to 60°C) to study the performance of laccase.

Specific details related to each experiment are given in each corresponding section. All the experiments were carried out in milli-Q water in 250 mL conical flasks with 100 ml of sample in an INFORS HT - multitron standard shaking incubator (INFORS, Canada) at 150 rpm at a pH of 6.5 (real wastewater conditions - in wastewater treatment plants); unless specified for a particular experiment) and at 25±1°C (room temperature; unless specified for a particular experiment). At the end of each experiment, the enzyme activity was stopped using methanol (purity >99.8%, Fisher Scientific Ottawa, ON, Canada) and samples were stored at 4°C for DCF analysis.

2.3.1 Kinetics of laccase-mediated DCF degradation

Degradation kinetics (substrate based) was carried out for 50 µg L^{-1}, 500 µg L^{-1} and 5 mg L^{-1} of DCF with an enzyme activity of 50 U L^{-1} for 5 days. Sampling was carried out each hour for the first 12 hours, for the first day and each 24 hours for next 4 days. Moreover, varying DCF concentration (50 µg L^{-1}, 100 µg L^{-1}, 500 µg L^{-1}, 1 mg L^{-1}, 5 mg L^{-1} and 10 mg L^{-1}) was tested against 50 U L^{-1} of laccase to study the Michaelis–Menten kinetics.

The Michaelis-Menten kinetic parameters of the DCF degradation by laccase was determined by measuring the initial degradation rate of DCF at different concentrations (equation (1)) [23].

$$V = \frac{V_m[S]}{K_M + [S]} \qquad \text{----------} \quad (1)$$

Where, V (µg L-1/hour) is the catalytic reaction rate, V_m (µg $L^{-1}h^{-1}$) is the maximum rate of the reaction, [S] is the concentration of substrate, and K_M is the Michaelis–Menten constant.

However, Km and Vmax values were obtained by fitting the data to the Lineweaver-Burke plot, resulting from the Michaelis–Menten plot conversion. First and second order rate equations were used to compare the reaction kinetics at higher as well as environmentally relevant concentrations.

2.3.2 Effect of pH and enzyme activity on DCF degradation

The effect of pH on degradation was studied at pH varying from 2 to 12.5. The initial DCF concentration was 500 $\mu g\ L^{-1}$ and the enzyme activity was 50 U L^{-1}. Experiments were carried out for 5 hours. Standard solutions of 500 $\mu g\ L^{-1}$ DCF were prepared in pH adjusted milliQ water prior to the experiment.

Varying enzyme activities of 50, 100, 500, 1000, 1250, 1500 and 1750 U L^{-1} were also tested against 1000 µg L^{-1} of DCF to find out the optimal amount of laccase required to degrade DCF. For this particular experiment, the sampling was carried out at 0^{th}, 5^{th}, 18^{th} 24^{th} and 48^{th} h. Various kinetic models were also studied under varying enzyme concentration levels to determine the effect of enzyme concentrations on the degradation of DCF.

2.3.3 Effect of temperature on DCF degradation

The effect of temperature on DCF degradation was carried out at varying temperatures from 10°C to 60°C with an initial DCF concentration of 500 µg L^{-1} for 5 hours and with an enzyme activity of 50 U L^{-1}. The experiments were carried out in milli-Q water in 250 mL conical flasks with 100 ml of sample in an INFORS HT–multitron standard shaking incubator (INFORS, Canada) at 150 rpm at a pH of 6.5.

2.4 DCF analysis and identification of transformation products

A newly developed and rapid Laser diode thermal desorption tandem mass spectroscopy (LDTD-MS/MS) was used for the analysis of DCF samples [6]. The instrument comprised LDTD-APCI (Laser diode thermal desorption - atmospheric pressure chemical ionization) source (LDTD T-960, Phytronix Technologies, Quebec, Canada) mounted on a TSQ Quantum access triple quadruple mass spectrometer (Thermo Scientific, Mississauga, Ontario, Canada). Potential transformation products were identified using LC-MS/MS. A detailed description for the quantification of DCF by this method is already given in a previous publication[6]. Transformation products (major) of DCF were

identified by scanning a mass range of m/z = 10-500. The obtained m/z values were compared with the previous studies reported the transformation products of DCF [24].

2.5 Statistical analysis

All the experiments for the production of laccase and degradation of DCF were carried out in triplicates. Data presented are the mean values with standard deviation (±SD). To investigate the statistical significance of the measured values of the standard curve, one way ANOVA test has been carried out with 95% confidence level using Statistica, version 11.0 (StatSoft, USA) and the results which have $p < 0.05$ were considered as significant. Moreover, to study the effect of various inducers, Tukey HSD test has been carried out.

3. Results and Discussion

3.1 Laccase production

3.1.1 Apple pomace, alfa fibers and pulp and paper solid waste as fermentation substrates

Figure 1 presents the laccase production profile over time using apple pomace, PPSW and alfa plant fibers. In comparison with alfa fibers, apple pomace and PPSW were effective substrates for the production of laccase. Without the use of any inducers, maximum production of laccase obtained by apple pomace was 36.4±4.7 U/gds (units per gram of dry substrate) on the 7th day; whereas for alfa, it was 9.15±1 U/gds on the 9th day (Fig 1). Without the use of inducers PPSW showed a maximum production of 34±3.6 U/gds on the 11th day. Apple pomace has been already well established for the production of various enzymes and platform chemicals [12, 25-26]. Previous studies reported that AP contains about 128 g Kg^{-1} of total carbon, 6.8 g Kg^{-1} of total nitrogen and 23.5 g Kg^{-1} of lignin[27]. Moreover, AP is a rich source of many micro and macro nutrients, such as Ca, Cu, Fe, K Zn etc.[28] and which makes AP as one of the best available agro-industrial residue for ligninolytic enzyme production[27-28]. PPSW is already proven to be a substrate for the production of several organic

acids through fermentation, such as fumaric acid.PPSW contains about 410 g Kg^{-1} of total carbon and 31.9 g Kg^{-1} of total nitrogen . In addition, previous studies reported rich presence of micro and macro nutrients, such as Mg, Ca, Fe, K, Zn, Mn, P etc. in PPSW[14]. Its been already proved that metal ions including Mg^{2+}, Zn^{2+} and Fe^{2+} act as cofactors for many cellular enzymes. Thus, PPSW can be a suitable substrate for enzyme production . Alfa fibers contain about 21.8% total lignin, about 45 % carbon and 1.3% nitrogen[18]. Thus, alfa fibers have promising potential for ligninolytic enzyme production which needs to be studied. The richness of nutrients and lignocellulosic content in apple pomace [27] in comparison with alfa plant fibers and the presence of phenolic compounds in apple pomace [29] which are essential for laccase production resulted in higher production of laccase. Optimal C/N ratio and the presence of several micro and macro nutrients including metal ions resulted in better laccase production from PPSW. The absence/ low concentration of these elements; micronutrients, such as Cu in particular made alfa plant fibers not suitable for higher laccase production[30]. Furthermore, none of the previous studies reported the use of alfa plant fibers as a potential substrate for enzyme production. To the best of our knowledge, this is the first study to report the same although the production was rather limited, which could be amended by supplementary nutrients.

3.2 Effect of various inducers

Various inducers were used to enhance the production of laccase and their effects have been given in table 1. The effect of inducers was minimal on overall laccase production. Of all the inducers used with apple pomace, copper sulfate (improved production by 35.2%) and veratryl alcohol (an increase in activity by about 28%) exhibited enhanced efficiency for production of laccase. Moreover, all inducers combined did not result in an enhanced laccase production. Previous studies also reported the applicability of veratryl alcohol and tween 80 as inducers for the production of various ligninolytic enzymes including laccase [19, 27]. Copper sulfate was reported to be an effective inducer for laccase production by *T.versicolor* [31] which was in accord with the current study. In addition, with apple pomace, maximum laccase production was observed on the 7th day of fermentation where as for

PPSW maximum production was observed on 11th day of fermentation. Both veratryl alcohol and copper sulphate exhibited enhanced production capacity with PPSW. With alfa plant fibers, all inducers, tween 80 (29 %), copper sulfate (56 %) and veratryl alcohol (48 %) demonstrated competence for enhanced laccase production. Maximum laccase production was observed on the 9th day of fermentation as compared with apple pomace (7th day). Similar to apple pomace, all inducers in the combined form did not result in an enhanced laccase production with alfa plant fibers. Analysis of variance was carried out at day 9 for alfa , day 7 for pomace and day 11 for pulp and paper solid waste which corresponded to respective maximum laccases production. Results indicated that use of inducers significantly affected the laccase production ($p<0.001$).Tukey posthoc analysis was used to compare between all inducers used (tables 1,2,3,4,5 and 6 in supplementary data). For alfa fibers, a significant increase in laccase production with copper sulfate was observed followed by phenol red, while no significant increase was observed when veratryl alcohol and tween 80 were used. For pomace, best inducers that can be used for significant improvement in laccase production was copper sulfate followed by veratryl alcohol. For both tween-80 and phenol red, no difference was observed when compared to the control ($p>0.05$). For pulp and paper solid waste , best inducers that can be used for significant improvement in laccase production was copper sulfate followed by veratryl alcohol.

From experiments and statistical analysis, copper sulfate was found to be the best inducer among the 4 inducers for alfa, apple pomace and pulp and paper solid waste . Laccases are blue copper oxidases and contain four copper atoms per molecule. Thus, copper is an essential element and addition of copper might lead to activating the metal and hence gene expression encoding laccase production [27, 32]. Tween-80 being a surfactant,[33] enhanced extracellular enzyme production by promoting both the uptake and release of compounds from the cells through the modification of plasma membrane permeability [27, 33]. Veratryl alcohol is a secondary metabolite known to have a significant role in the degradation of lignin as cation radical mediating redox reactions[34]. It is reported that laccase production can be stimulated by a wide variety of inducing substrates composed of

aromatic or phenolic origin those are related to lignin or lignin derivatives such as ferulic acid, 2,5-xylidine, and veratryl alcohol [27, 34]. There an increasing trend in production until the 7th day for apple pomace and 9th day for alfa during fermentation and afterward the laccase activity tapered off. Multiple reasons can be attributed towards this decrese in laccse activity. Lignocellulosic materials are already present in the substrate. The produced enzyme could be used for the degradation of this lignin and could be deactivated. Moreover, availability of nutrients decreased with fermentation time which affected the laccase production as laccase production is growth dependent. Another potential reason was the changes in pH during fermentation[35-36] as the experiments were conducted in batch scale in flasks without pH control. Due to metabolic acivities of *T.versicolor* and hence the degradation of substrate, changes in pH were observed in the medium. These changes in pH can further affect the laccase production.

This study presents few substrates that can be potentially cost-effective for laccase production. In the present study, the residuals are used as sole substrates and they make up for the 40-60% cost in the total production scale as also proved in our earlier studies of our research group[37]. The best results were observed with PPSW and apple pomace. From the information gatherned from the respective industries, apple pomace costs about $100/tonne and PPSW cost about $20/tonne. Potato dextrose agar (PDA) supplemented with phenolic compounds can be considered as the standard media for laccase production and which costs about $1000/tonne for bulk purchase (Alibaba.com). Moreover, basal medium was also reported as an effective medium for laccase production[38], however, it is cost intensive. Thus, PDA was considered as the standard medium for these approximate cost estimations. All the expenses related to fermentation (sterilization, incubation, extraction etc..) for the standard media and the residual materials used for e this study are the same. Hence, they were not considered and thus approximate cost was calculated based on media cost. Therefore, as an approximation, while using apple pomace, a 90% reduction and while using PPSW, a 98% reduction in expenses was observed. Moreover, under optimal conditions, one

tonne of PPSW can produce 51 x 10^6 IU (international units) of laccase whereas, apple pomace can produce 49 x 10^6 IU of laccase.

3.3 Degradation of DCF

3.3.1 Degradation kinetics

The kinetics of DCF degradation over time was studied at different concentrations such as 50 µg L^{-1}, 500 µg L^{-1} and 5 mg L^{-1}. The removal efficiency of laccase for DCF over time is presented in Figure 2.

DCF degradation followed first-order kinetics at very low concentration of 50 µg L^{-1} (R^2= 0.95) and had some agreement with zero order (R^2 = 0.94) and first order (R^2 = 0.83) at intermediate concentration of 500 µg L^{-1}. Pursuing the same trend, the rate of degradation exclusively followed zero order (R^2 = 0.92) at a higher concentration of DCF i.e. 5 mg L^{-1}. Further, DCF degradation followed Michaelis-Menten kinetics having a parabolic curve from 50 µg L^{-1} to 5 mg L^{-1}. This further confirmed the order of reaction of DCF degradation since in Michaelis-Menten kinetic model, the degradation rate is zero order at higher substrate concentrations (for diclofenac- in comparison with real environmental conditions) and first order at lower substrate concentrations (for diclofenac- in comparison with real environmental conditions). Moreover, this also confirms the inhibition at higher DCF concentrations due to the shift from first order to zero order. In addition, Lineweaver-Burke model was also plotted and this also showed excellent agreement with the kinetic data. The plot was fitted with an R^2 of 0.98. The equation obtained from Lineweaver-Burke plot[39] was used to calculate K_m and V_{max} of the degradation process. V_{max} was found to be 80.6 µg L^{-1} $hour^{-1}$ and K_m was calculated to be 2675 µg (data not shown). It has been reported that the degradation of micropollutants followed first order with peroxidases [40] degradation due to low concentrations, such as few millimolar levels. Moreover, laccase-mediated degradation of textile dyes and few endocrine disrupting compounds respected first order degradation following Michaelis-Menten principles[23, 41-42]. However, to the best of our knowledge, kinetics and rate of degradation of DCF with fungal

laccases have never been reported earlier. Thus, considering the µg L^{-1} levels of DCF [2, 6] in wastewater, DCF will follow first order kinetics with a maximum velocity of 80.6 µg L^{-1} $hour^{-1}$. As a result, degradation using fungal laccases can be an excellent biological removal method for DCF. Throughout this study, the concentration of DCF was chosen resembling environmentally relevant concentrations. In addition, other experimental conditions, such as pH have been carefully selected to match with real wastewater conditions. Thus, the obtained velocity of degradation (80.6 µg L^{-1} $hour^{-1}$) can provide substantial insight while using laccase for the degradation of DCF in real environmental conditions.

3.3.2 Temperature and pH

Temperature and pH were two crucial factors for laccase-mediated DCF degradation. The degradation trend of DCF along with pH and temperature is given in Figure 3.

From fig.3, it is clear that the maximum degradation of DCF occurred at pH 4 and hence it was the optimum pH for degradation. Again, an increase in degradation was observed at pH 10 and which further decreased along with an increase in pH. Thus, the maximum degradation was observed at pH ranging from 4-5. It is reported that for ABTS, which is the standard substrate used for the determination of laccase activity, maximum substrate conversion was observed at pH 4.5 [20]. The pKa of DCF (4.15) can be directly correlated with the efficient degradation of DCF at pH 4.5. Previous studies also reported pH 3-5 as effective for the DCF degradation[43] Moreover, it was reported that laccases from fungal origin work efficiently at acidic pH[44]. Enzyme activity of laccases may decrease at higher pH because of the formation of hydroxide anion. Hydroxide anion will bind to the T2/T3 coppers of laccase and which in return disturb/block the internal electron transfer from T1 to T2/T3[45].

For laccases, degradation of DCF was increased along with temperature. It has been reported that optimal temperature for pollutant degradation largely depends upon the pollutant characteristics, such as molecular structure, pKa and solubility[43]. Laccase-mediated reactions are largely

temperature dependent in which rate of reaction is often controlled by temperature due to Arrhenius effects[46]. It has been reported that laccase is active at a higher temperature and the standard assay for laccase is being carried out at 45°C. Moreover, for DCF, the solubility can be increased at elevated temperatures and as a result which will increase the availability of DCF for laccase-mediated catalysis. Thus, as shown in fig.3 (B), increasing temperature had a positive effect on DCF degradation. Previous studies reported that laccase-catalyzed de-colouration various synthetic azo dyes such as acid blue 92 followed the same pattern of degradation along with temperature[47].

3.3.3 Reaction rates - varying enzyme activities

The obtained enzymes from SSF through apple pomace were further concentrated using ultrafiltration so that the impurities derived from the substrate AP was removed. The effect of enzyme activity on DCF degradation is given as Figure 4. The experiments were carried out using 1 mg L^{-1} of DCF. More than 90% degradation for DCF was obtained with an enzyme activity of 500 U L^{-1} in 5 hours. At the 24th hour, it reached nearly 100 %. Further, this trend has been observed with increasing laccase concentrations. With enzyme activities of 1000, 1250, 1500 and 1700 U L^{-1}, respective DCF removal of 96.6 %, 97.5%, 98% and 98.3 % was observed. In all these cases, at 18th and 24th hour, a removal of ca.100 % was observed. A similar degradation was observed for DCF using T. *versicolor* pellets [24]. The influence of the enzyme activity on the transformation rate was not directly proportional. Thus, an enzyme activity of 500 U L^{-1} can be considered effective for DCF degradation. Further increasing the laccase activity did not result in the enhanced removal.

3.3.4 Transformation products and reaction pathways

To find the degradation pathway of DCF treated with 1500 U L^{-1} of laccase, the reaction mixture was analyzed at 5th, 18th and 24th hour of degradation. Samples were analyzed using both LDTD-MS/MS and LC-MS/MS instruments with non-targeted scanning to find out the clearly visible peaks and corresponding *m/z* values. Previously optimized methods were used for the analysis of DCF[6]. It was observed that non-targeted scanning with LDTD-MS/MS did not result in the accurate identification

of peaks. Thus, LC-MS/MS was applied owing to its chromatographic separation technique. Peaks with a relative peak area of more than 4 were only considered while searching for metabolites. At 5th hour of degradation which corresponds to 98 % DCF degradation, m/z of 312.14 (highest peak area), 327, 296 and 260 was observed. At 24 hour, no peaks with the significant area were observed and this can be interpreted as complete degradation.

From the molecular weight of the compounds observed, m/z 312 corresponds to 4′-Hydroxydiclofenac, 5′-Hydroxydiclofenac or 3′-Hydroxydiclofenac. Likewise, m/z 296 corresponds to the diclofenac ion. Similarly, m/z 327 corresponds to 4′,5-dihydroxydiclofenac The molecular structures of these compounds are given in table 2. However, authors were not able to exactly identify the molecular structure corresponding to 260. Further, NMR analysis is required to exactly identify this metabolite.

Many of these metabolites were previously described in biodegradation of DCF; mostly when fungi and bacteria were used to synthesize hydroxylated diclofenac, which is of interest in the pharmaceutical industry. Previous studies described the production of 4′-OH diclofenac and small amounts of 3′-OH and 5-OH diclofenac using filamentous fungi. Moreover, a larger shift in the peak intensity at –OH region was observed with transformation products using FT-IR analysis (the FTIR spectrum is given as supplementary information). Surface functional groups of the microparticles were identified by Nicole iS50 FT-IR Spectrometer (Thermo Scientific, USA).This shift can be related to the formation of -OH substituted compounds and thus confirms the presence of hydroxyl substituted transformation products of DCF. Conversion of diclofenac to 4′-OH diclofenac using an extracellular peroxigenase of the basidiomycete *Agrocybe aegerita* was reported earlier [48]. Metabolites 1,2 and 3 are the most widely reported metabolites for DCF and are human metabolites as well [2]. No peaks were observed in LC-MS/MS spectrum after 24 hours of degradation of DCF. Thus, neither DCF nor any specific transformation products can be identified. This suggested that degradation mechanism can be proposed as hydroxylation followed by ring opening and final mineralization to CO_2, NH_3, HCl and H_2O. The proposed pathway is given as figure 5.

This study provides novel insight on waste valorization and treatment of emerging contaminats. Cost-effective production of of laccase was achieved through the utilization of residual materials as substrates. Ecological advantages include waste management, green process, waste valorization and sequestration of waste carbon leading to reduced carbon footprint and hence mitigation of greenhouse gases. Moreover, produced laccase was utilized for the degradation of emerging contaminant, diclofenac and optimal degradation conditions were identified.

4. Conclusions

Apple juice industry residue, apple pomace (49.16±2.5 U/gds)) and pulp and paper solid waste (52.4±2.2 U/gds) were found to be the efficient substrates for laccase production over dried alfa fibers (14.26±0.8 U/gds). Copper sulfate was found to be an efficient inducer for laccase production over tween 80, veratryl alcohol and phenol red. However, the inducing effect of the studied inducers were rather limited with all substrates. At environmentally relevant concentrations (500 µg L^{-1} of DCF), pH of 4.5 and temperature of 50 °C was found to be optimal for the effective degradation of DCF with laccase. For laccase-mediated degradation, the rate of reaction was found to be zero order at higher substrate concentrations and first order at lower substrate concentrations (environmentally relevant). Under favorable conditions, up to 99 % degradation was observed for DCF within 5 hours. The initial transformation products were identified by LC-MS/MS and identified as 3′-hydroxydiclofenac, 4′-hydroxydiclofenac, and 5-hydroxydiclofenac. Laccase-catalyzed DCF degradation mechanism has been proposed as hydroxylation ring opening and final mineralization to CO_2, NH_3, and H_2O and chlorine.

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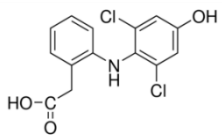
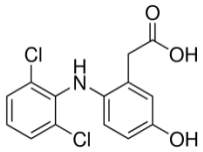
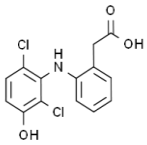
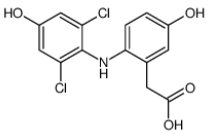
Table 1: Effect of various inducers on laccase production

Inducer	Time (day)	Laccase production (U /g of dry substrate)		
		Apple pomace	Alfa plant fibers	Pulp and paper solid waste
Without inducers	5	32.8±3	5.65±0.1	17.2±2.8
	7	36.4±4.7	6.4±0.3	19.7±2.2
	9	22.9±1.5	9.15±0.9	33.1±2.6
	11	0.61±0.1	4.3±0.2	34±3.6
	14	0.4±0.1	1.2±0.1	7.6±0.5
Tween 80	5	20.4±1.6	5.4±0.25	12.5±0.9
	7	38.3±5.1	6.33±0.6	24±0.5
	9	27.4±2.4	11.8±0.6	34.1±3.5
	11	3.63±0.2	5.6±0.4	37.6±3.3
	14	1.54±0.2	0.9±0.1	12.6±3.6
Veratryl alcohol	5	28.6±2.5	9.7±0.7	20.2±0.1
	7	46.6±2.5	9.49±0.4	27.8±7.6
	9	29.8±1.6	8.26±0.7	32.8±6.5
	11	5.78±0.9	7.2±0.6	51.2±1.6
	14	2.65±0.02	1.1±0.2	16.6±1.6

	5	32.49±2.8	7.7±0.7	14.4±0.8
	7	49.16±4.5	9.49±0.4	40.8±.1
Copper	9	14.16±2.3	14.26±0.8	51.2±0.5
sulfate	11	4.32±1.1	7.2±0.4	52.4±2.2
	14	1.66±.0.8	3.1±0.2	9.2±1.6
	5	19.6±2.8	4.1±0.4	32.2±0.8
	7	27.8±0.5	8.8±0.5	42.9±.0.7
Phenol red	9	42±8.4	15.3±1.8	26.6±0.1
	11	21.2±1.6	15.8±1.1	25.8±1.3
	14	10.3±.0.9	3.4±0.8	3.1±1.5
	5	19.04±1.4	4.3±0.6	11.2±0.5
	7	32±3.2	5.2±0.4	15.04±1.4
All inducers combined	9	24.4±3.2	8.26±0.9	20.4±3.7
	11	1.63±0.6	3.4±0.2	27±1.2
	14	1.57±0.1	0.8±0.1	9.6±0.5

Table 2: Diclofenac major transformation products- initial hours

Transformation product	Molecular structure	First reports on transformation products
4'-hydroxydiclofenac		49-51
5'-hydroxydiclofenac		49-51
3'-hydroxydiclofenac		49, 51
4',5-dihydroxydiclofenac		49, 51

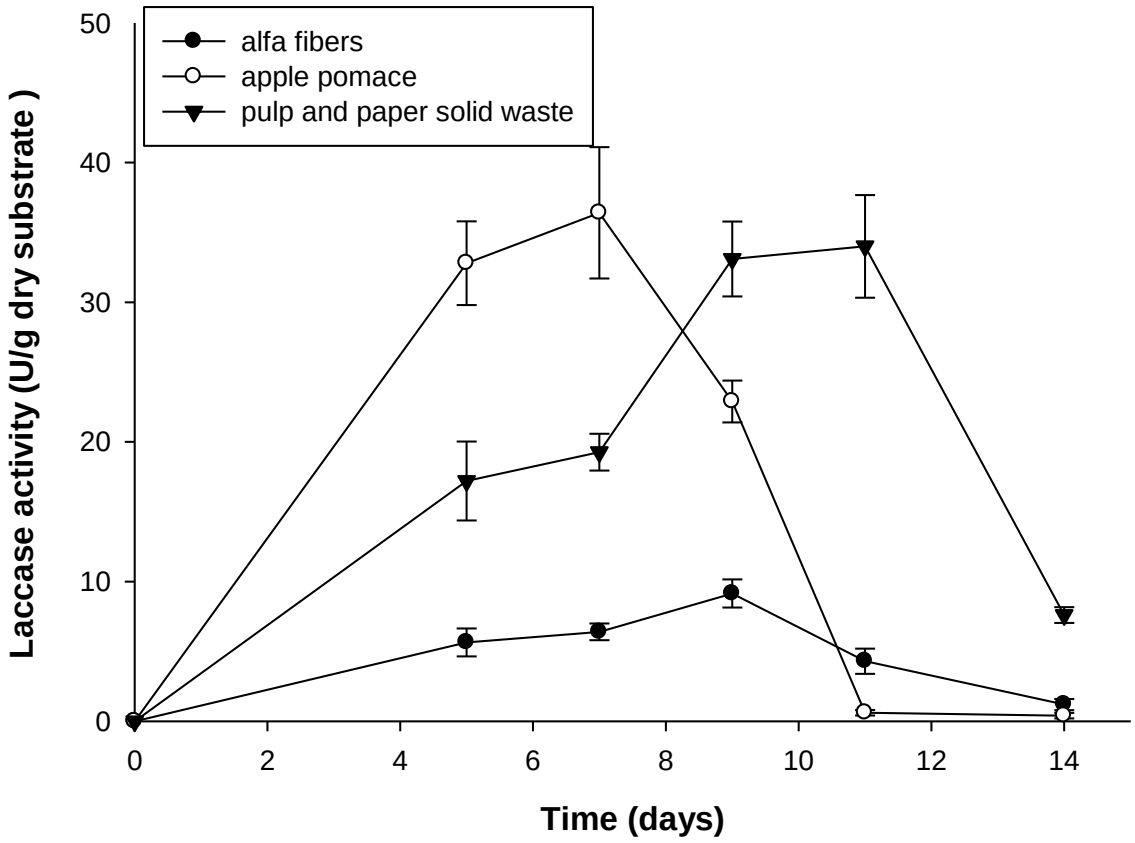


Figure 1: Production of laccase using alfa plant fibers, apple pomace and pulp and paper solid waste as substrates

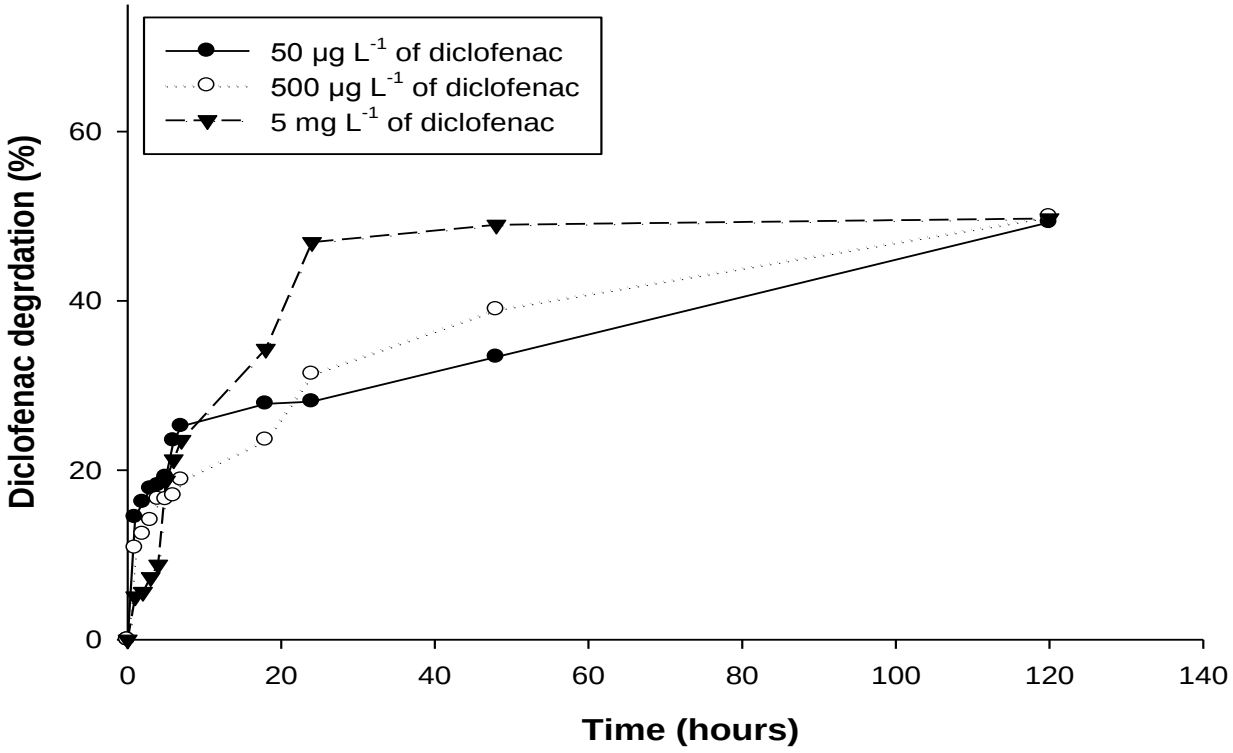


Figure 2: Diclofenac degradation over time, laccase activity = 50 U L^{-1}; pH = 6.5

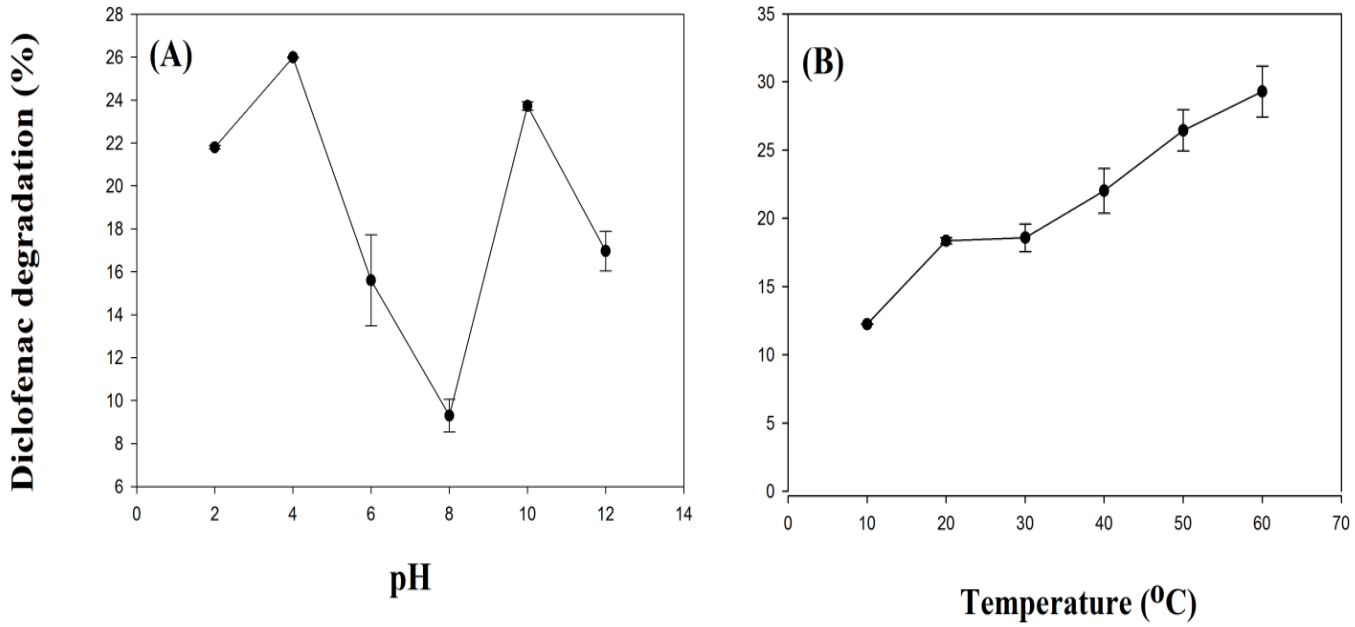


Figure 3: Laccase mediated diclofenac degradation, (A) with pH (at a constant temperature, 25 °C); (B) with temperature (at a constant pH , 6.5) ; diclofenac concentration = 500 µg L^{-1}, enzyme activity = 50 U L^{-1},

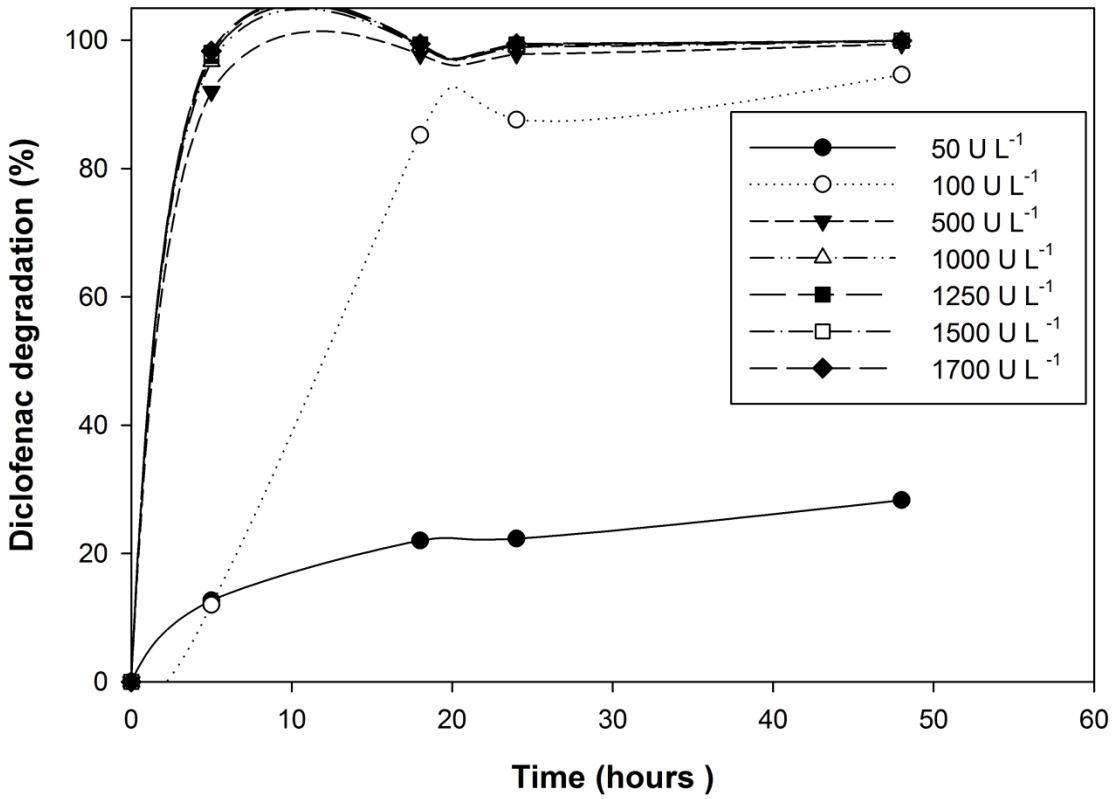


Figure 4: Laccase mediated diclofenac degradation at various laccase activities; temperature =25 °C; pH = 6.5; diclofenac concentration = 1 mg L^{-1}

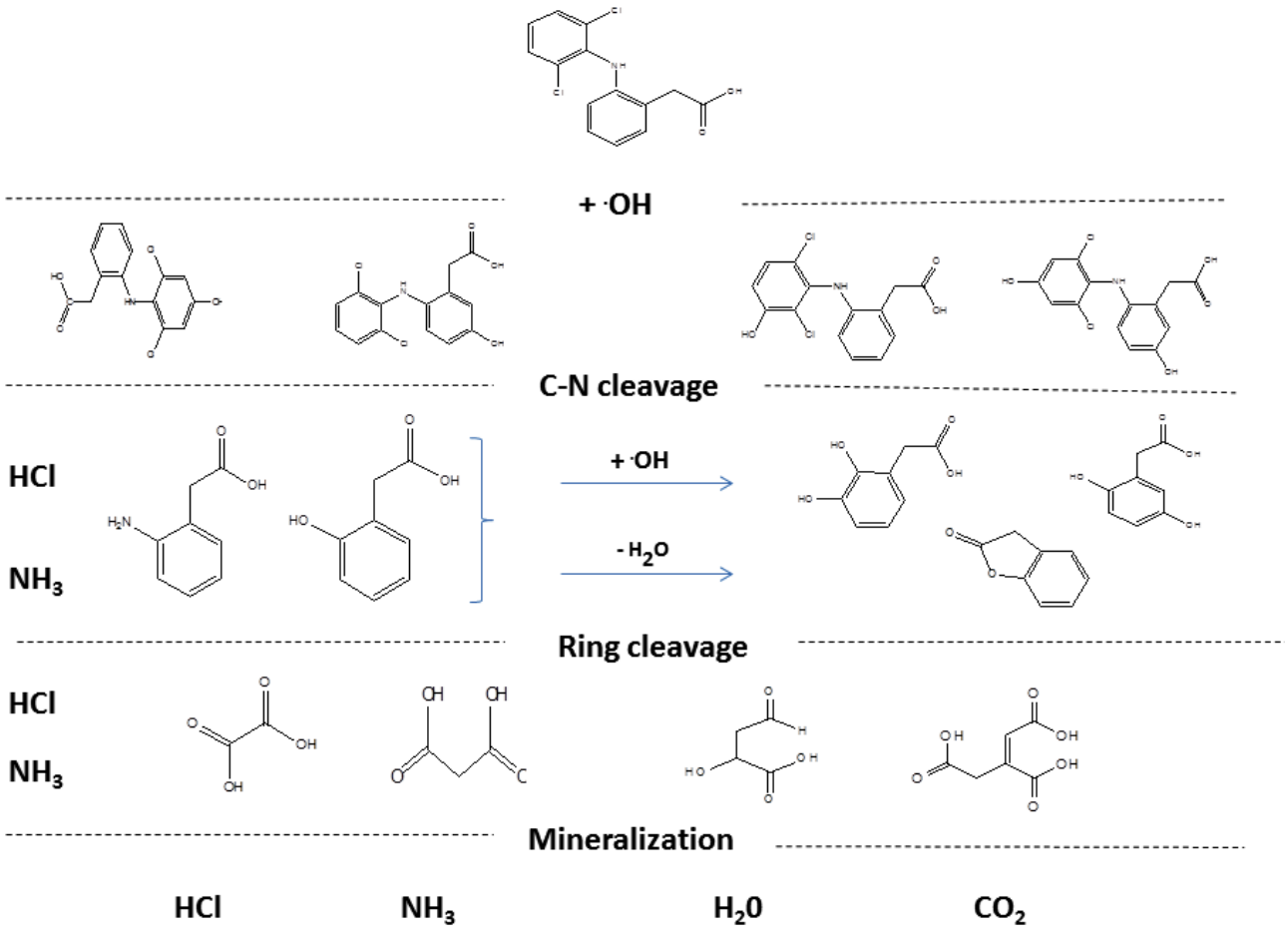


Figure 5: Proposed laccase mediated diclofenac degradation pathway

PART 2

ADSORPTIVE IMMOBILIZATION OF AGRO-INDUSTRIALLY PRODUCED CRUDE LACCASE ON VARIOUS MICRO-BIOCHARS AND DEGRADATION OF DICLOFENAC

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SCIENCE OF THE TOTAL ENVIRONMENT (UNDER REVIEW)

RÉSUMÉ

Dans cette étude, de la laccase brute produite agro-industriellement a été concentré par ultrafiltration et immobilisé sur des microparticules de biochars de bois de pin (BC-PW), de fumier de porc (BC-PM) et de coquille d'amandes (BC-AS). L'immobilisation de la laccase a été étudiée à différentes activités sur les microbiochars et la cinétique de libération (désorption) de l'enzyme a été mesurée. Le BC-PM s'est avéré être le plus efficace (31.4 ± 3.1 U g^{-1}) à 10 U mL^{-1} d'activité enzymatique, suivi du BC-AS (24.3±4.8 U g^{-1}) et du BC-PW (14.58±3.3 U g^{-1}). De plus, les microbiochars fonctionnalisé à l'acide citrique ont été étudié pour l'immobilisation de la laccase brute. Les études d'isotherme ont établi que l'adsorption en monocouche homogène est le mécanisme majeur. La désorption de la laccase a suivi une cinétique de pseudo-second ordre. La laccase immobilisé a démontrer une capacité à être entreposer (durée de conservation) trois fois plus élevée que la laccase libre. Finalement, la laccase immobilisée a été utilisée pour la dégradation du DCF, un important micropolluant, et près de 100% d'enlèvement a été obtenu après 5 heures avec des concentrations représentatives de celles retrouvées dans l'environnement.

Mots clés : Laccase, biochar; diclofenac; biotransformation; immobilisation adsorptive; isotherme, désorption

ABSTRACT

In this study, agro-industrially produced crude laccase was concentrated using ultrafiltration and immobilized on pine wood (BC-PW), pig manure (BC-PM) and almond shell (BC-AS) biochar microparticles. Immobilization of laccase was investigated at various laccase activities on micro-biochars and the release kinetics (desorption) of the enzyme has been studied. BC-PM was found to be the most effective (31.4±3.1 U g^{-1}) at 10 U mL^{-1} of enzyme activity followed by BC-AS (24.3±4.8 U g^{-1}) and BC-PW (14.58±3.3 U g^{-1}). In addition, citric acid functionalized micro-biochars were studied for crude laccase immobilization. Isotherm studies established homogeneous monolayer adsorption as major mechanism. The desorption kinetics of laccase followed pseudo-second-order. Immobilized laccase exhibited superior storage ability/ shelf-life which were three times higher than free laccase. Finally, the immobilized laccase was used for the degradation of micropollutant, DCF and near 100% removal was obtained within 5 hours at an environmentally relevant concentration (500 µg L^{-1}).

Keywords: Laccase; Biochar; Diclofenac; Biotransformation; Adsorptive immobilization; Isotherms; Desorption

1. Introduction

Gifted with unique unprecedented catalytic activity and selectivity over a wide range of pollutants, enzymes offer tremendous potential for environmental remediation. In particular, ligninolytic enzymes are well known for degradation of recalcitrant pollutants (Tien & Kirk, 1983), (Majeau et al., 2010; Strong & Claus, 2011). Along with manganese peroxidase and lignin peroxidase, laccases are known to be the prominent enzymes in this category due to its wide substrate range, simple requirements for catalysis, apparent stability and lack of inhibition in comparison with other enzymes (Majeau et al., 2010). However, low thermal and pH stability (narrow pH range in activity), loss of catalytic activity after one cycle and huge consumption loss while being used in water environment have remained as major obstacles in large-scale environmental applications (Jesionowski et al., 2014). Thus, past studies investigated the improvement of enzyme stability and their properties for environmental applications, and immobilization of enzymes on solid supports was suggested as an effective method (Cowan & Fernandez-Lafuente, 2011; Mateo et al., 2007).

Immobilization of enzymes on solid supports is primarily based on two approaches: physical binding and covalent binding between the enzyme and the supports. Physical binding (includes van der Waals forces, ionic interactions, and hydrogen bonding) through adsorption is often considered as the most economically viable method (Jesionowski et al., 2014). Enzyme-support affinity is the most important factor in adsorptive immobilization along with a larger surface area. The presence of specific active functional groups on the surface of the carrier along with a large surface area of the carrier can enhance the adsorptive immobilization of enzymes on solid supports. Recently, carbonaceous material, such as biochar is getting wide attention due to its great potential in various domains including agricultural applications and environmental remediation (Glaser et al., 2009). The possibility of production from waste materials, moderate surface area and abundant presence of surface functional groups make biochar an excellent adsorbent for various contaminants (Fang et al., 2014; Lonappan et al., 2017a). Also, due to these dependable properties, biochars can be used as immobilization support for laccases.

Diclofenac (DCF), being an emerging micropollutant poses toxic concerns towards several aquatic as well as terrestrial organisms (Lonappan et al., 2016a; Vieno & Sillanpää, 2014). Therefore, DCF must be removed from the environmental compartments, for a sustainable and healthy environment and ecosystem. Previous studies have demonstrated the efficiency of laccase for the degradation of DCF (Lonappan et al., 2017b; Marco-Urrea et al., 2010). Nevertheless, these studies were mostly focused on the production and application of free laccase for the degradation of DCF and thus, incurred disadvantages of application of free enzyme limiting their applicability at large scale and in long-term. Therefore, the possibility of immobilizing crude non-purified laccase produced from agro-industrial materials (most economically convenient production method as well as through valorization of waste materials) on micro-biochar (efficient, least expensive and valorization of waste materials) derived from different feedstocks was investigated in this study. This approach has the dual advantage of continuous adsorption as well as degradation of the contaminants (DCF in this study). Moreover, this study employed the simple adsorption technique for immobilization which can be considered as green as it avoids usage of any chemicals. Immobilization of laccase at various concentrations on various biochars and the release of the enzyme have been studied. In addition, the biochars were functionalized with organic acids for possible surface modifications, and their effect on adsorption of enzymes has been investigated. Finally, the laccase-immobilized biochars were applied for the degradation of DCF in batch mode under various experimental conditions and the results are presented. In brief, the immobilization of non-purified laccase on organically functionalized biochars (and non-functionalized) along with its potential application for the degradation of model micropollutant presents the novelty elements of this study.

2. Materials and Methods

2.1 Materials

Pinewood and almond shell biochar samples were obtained from Pyrovac Inc. (Quebec, Canada). Pinewood biochar (BC-PW) was derived from pine white wood (80% v/v) spruce and fir (20% v/v)

and was produced at 525±1 °C under atmospheric pressure by increasing the temperature of biomass at the rate of 25 °C min^{-1} in the presence of nitrogen. Almond shell biochar (BC-AS) was produced from almond shells slightly under atmospheric pressure at 520±1 °C for 4 h. Pig manure biochar (BC-PM) was a gift sample from Research and Development Institute for Agri-Environment (IRDA), Quebec, Canada. BC-PM was derived from the solid fraction of pig slurry and prepared at 400±1 °C for 2 h.

Juice industry residue, apple pomace was used as the substrate for the microorganism for enzyme production which was gifted by Vergers Paul Jodoin Inc., Quebec. White-rot fungus, *Trametes versicolor* (ATCC 20869) was used as the microorganism for the production of laccase.

Diclofenac sodium salt (98%; CAS 15307-79-6) was purchased from Fisher Scientific (Ottawa, ON, Canada) and internal standard (IS), diclofenac-d_4 was obtained from C/D/N Isotopes Inc. (Montreal, QC, Canada). All other chemicals used in this study (including organic acids used for the functionalization of biochars) were of analytical grade and they were obtained from Fisher Scientific and Sigma-Aldrich (Ontario, Canada).

2.2 Methods

2.2.1 Pre-treatment of biochar

All the biochars were ground and sieved to obtain biochar microparticles less than 75 µm. This pre-treatment produced compact, almost uniformly sized (distributed) biochar with an increased surface area. Functionalization procedure of biochar microparticles is given in previous publication (submitted)(Lonappan et al., 2018). In short, the micro-biochars were shaken with 2M citric acid for 24 hours. Then, the samples were centrifuged at 3000 x g and washed twice using deionized water. Further, micro-biochar samples were dried at 60°C and used for the immobilization of laccase.

2.2.2 Preparation of laccase

Detailed procedure for the production of laccase has been presented elsewhere (Lonappan et al., 2017b). In short, the fermentation substrates were adjusted to 75 ±1 % (w/w) moisture with sterile milli-Q water. Fermentation was carried out at 30 ± 1 °C for 9 d in 500 mL Erlenmeyer flasks containing 20 g of apple pomace. Extraction of laccase was carried out using 50 mM sodium phosphate buffer at pH 6.5 (10/1: *v/w*).

Crude laccase obtained by fermentation from apple pomace was concentrated by ultrafiltration using Sartorius stedim- SartoJet ultrafiltration system (Göttingen, Germany). Sartocon Slice Hydrosart Cassette- 10 kDa and 100 kDa were used as the filtration membranes. Harvested enzyme was filtered using 100 kDa membrane to remove the particles having size more than 100 kDa. Later, the filtrate was passed through 10 kDa and the retentate having particles of molecular weight less than 10 kDa have been discarded. This resulted in semi-purification of laccases.

2.2.3 Enzyme assay

Laccase activity was determined by measuring the rate of color generation due to the enzymatic oxidation of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) to ABTS radical ($ABTS^{+}$) at 420 nm and pH 4.5. The extinction coefficient (ε) used was 36,000 M^{-1} cm^{-1} (Wolfenden & Willson, 1982). The assay solution contained 100 mM citrate-phosphate as the buffer (pH 4.5), 0.5 mM ABTS and enzyme. Enzyme-ABTS mixture was incubated for 4 min and the average increase in absorbance per min was taken. One unit of laccase activity (U L^{-1}) is defined as the production of 1 µM $ABTS^{+}$ per min under assay conditions (Bourbonnais & Paice, 1992; Collins & Dobson, 1997).

2.2.4 Immobilization of laccase on biochars

Throughout the experiment, a constant solid: liquid ratio of 1: 5 (w/v) was maintained between biochars and enzyme extract. Various enzyme concentrations were tested for immobilization as the

highest enzyme activity obtained after concentration of enzyme was 10 U mL^{-1}. Biochars were mixed with crude enzyme using a vortex blender (Fisher Scientific 02215370 Vortex mixer-deluxe 120V) at 1200 rpm. Initially, the mixing was carried out for 5 h and in later experiments; the mixing was done for 12 h (overnight) to have maximum possible adsorption of enzymes on biochars. Thus, the effect of contact time and adsorptive immobilization was studied. Various enzyme concentrations of 0.5, 2.5, 5, 7.5, and 10 U mL^{-1} were studied by keeping the biochar amount constant. Similar experiments were repeated with the functionalized biochar obtained from pine wood, pig manure and almond shell. Citric acid was used for the functionalization of micro-biochars. After immobilization experiments, enzyme- biochar mixture was centrifuged at 6000 x g and the liquid portion was removed. The solid fraction was gently washed using deionized water, later centrifuged and the liquid part was removed. Then, the remaining enzyme in the liquid was analyzed for enzyme (activity) and hence effective enzyme immobilized on biochar was calculated in U g^{-1}. Enzyme- immobilized biochar was dried at room temperature/ or by using compressed air carefully without increasing the temperature above 28±1 °C. Moreover, 0.1 g of enzyme immobilized biochar (dried) was shaken for 15 min with 10 mL of sodium phosphate buffer at pH 6.5 and the enzyme activity in the supernatant was measured after centrifugation. This procedure was carried out to make sure the immobilization of enzyme on biochar surface; however, the values were not used for calculation due to the variations in release kinetics from different biochars.

Studies on release of enzymes (desorption) from biochars were carried out under shaking conditions over time for two days (at pH 6.5). The stability of enzyme- immobilized biochar (shelf life) was tested after 3 months. In short, free and immobilized laccase were stored at 4± 1 °C for up to 60 days and their activities were determined in 20 days interval.

2.2.5 Characterization of laccase immobilized biochar

Particle size distribution was measured using a “Horiba particle size analyzer “(LA-950). Mean values were taken after analyzing the samples twice. Surface morphology of micro-biochar and

enzyme immobilized micro-biochar was investigated using an "EVO® 50 smart scanning electron microscope" (SEM) (Zeiss, Germany). The specific surface area of micro-biochars before and after laccase immobilization was obtained from Brunauer, Emmett and Teller (BET) N_2 adsorption isotherms at 77 K (Autosorb-1, Quantachrome, USA). Analysis on changes in surface functional groups of micro-biochars before and after laccase immobilization was carried out using Fourier transform infrared (FT-IR) spectrometer (Nicolet iS50, Thermo Scientific, USA).

2.2.6 Diclofenac degradation experiments

Laccase-immobilized biochars (most suitable sample from each micro-biochar) was tested for the degradation of DCF. The degradation experiments were carried out at room temperature and at pH 6.5. About 10 mL of 500 µg L^{-1} DCF was used as the initial concentration. About 0.5 g of laccase immobilized biochar was used for the experiments. Experiments were carried out for 12 h with continuous sampling. Supernatant from the samples was collected and mixed with equal amount of methanol (purity >99.8%, Fisher Scientific Ottawa, ON, Canada) to stop enzyme activity. The samples were stored at 4 °C and later analyzed for DCF using LDTD-MS/MS (Lonappan et al., 2016a).

3. Results and discussion

3.1 Laccase: concentration by ultrafiltration and enzyme activity

The major focus of this study was on adsorptive immobilization of crude laccase on micro-biochars from various sources. Laccase was produced using agro-industrial residue, apple pomace as the substrate for microorganism (Lonappan et al., 2017b). Possible presence of several other biomolecules can be expected in these crude extracts since extraction of the enzyme was carried out from fermented apple pomace. These biomolecules/unwanted substances can include phenolic substances, various organic acids, sugars and minor fractions of minerals lipids and proteins (Gassara et al., 2011; Persic et al., 2017; Sato et al., 2010). Various concentrations of laccase

(based on enzyme activity, U mL^{-1}) were selected and laccase was concentrated using ultrafiltration by 10 and 100 kDa membranes following previous reports on the molecular weight of fungal laccases (60-100 kDa) (Majeau et al., 2010). The filtrate after passing through each membrane was analyzed for enzyme activity to measure the efficacy of the ultrafiltration. Maximum of 10 U mL^{-1} of laccase was obtained at the end of concentration from an initial activity of about 0.8 U mL^{-1}.

3.2 Characterization of laccase-immobilized biochar

Particle size distribution of biochar microparticles was well below 75 µm as expected (data not shown). SEM images of raw and enzyme immobilized biochars (samples with best enzyme loading) is given as Fig. 1. The porous surface texture was clearly visible, however, any significant differences in the surface texture were not observed after immobilization of laccase. The possible reason could be the fact that enzymes are not visible through SEM owing to their small size. A similar phenomenon was observed in the SEM images reported by a previous publication (Qiu et al., 2008). Fig. 2 presents the FTIR spectra of micro-biochars before and after laccase immobilization. Clear and distinct peaks of laccase are not visible, after immobilization. This could be due to the fact that the concentration of laccase was very small to observe a peak while comparing with biochar. The surface areas of micro-biochars used were 14.1 m^2 g^{-1}, 46.1 m^2 g^{-1} and 17 m^2 g^{-1} for BC-PW, BC-PM and BC-AS, respectively. After laccase immobilization, the area decreased to 1.89 m^2 g^{-1}, 1.46 m^2 g^{-1} and 1.24 m^2 g^{-1} in the respective order. About 86.6% decrease in surface area was observed for pine wood biochar, whereas almost 97 % of the surface of pig manure biochar was covered with the molecules which were present in the crude enzyme mixture. In the case of almond shell biochar, reduction in surface area after laccase immobilization was 92.7%. This larger decrease in surface area provided the quantitative evidence for the laccase immobilization through surface adsorption. However, this cannot be attributed solely towards laccase adsorption since other bio-molecules/impurities having molecular size between 10-100 kDa were also present in the crude extract. Similar observation in reduction of surface area after enzyme immobilization was observed in many previous studies as well (Badgujar et al., 2013; He et al., 2006; Naghdi et al., 2017; Pirozzi

et al., 2009).For example, up to 89.1% decrease in surface area was observed in an immobilization study of lipase on mesoporous silica(He et al., 2006).

3.3 Adsorptive immobilization of laccase onto micro-biochars

Biochar properties including surface characteristics vary according to the feedstock as well as the pyrolysis type(Fang et al., 2014; Liu et al., 2015). In this study, three different types of biochars, namely, BC-PW, BC-PM, and BC-AS were selected to understand the effect of surface features and chemical composition of biochars on immobilization of laccase and identify the best feedstock. Moreover, it is already established that reduction in particle size can increase effective surface area (Lonappan et al., 2016b) and thus the micro-biochar was prepared and used. It has also been reported that the presence of carboxylic acids on the support can enhance immobilization of enzymes (Cho & Bailey, 1979). Thus, carboxylic acids functionalized biochar (through citric acid) was tested for the improved immobilization of laccase.

Fig. 3 presents laccase binding on non-functionalized biochars. Various laccase concentrations, (enzyme activities),such as 0.5, 2.5, 5, 7.5, and 10 U mL^{-1} were used for the immobilization of laccase on micro-biochars. It was observed that binding increased as the initial laccase activity increased. This observation was unanimous in all the three micro-biochars. This phenomenon can be explained based on adsorption. As the initial activity of laccase increased, more laccase molecules were available in the solution. This increased the effective interactions between micro-biochar and laccase molecules and hence better adsorption leading to higher immobilization. The previous study on the immobilization of α-amylase onto bentonite/chitosan composite has reported increased adsorption of enzyme with increased initial concentration (Baysal et al., 2014). In addition, another study on immobilization of bovine catalase onto magnetic nanoparticle reported the same trend in loading the enzyme onto the nanoparticles. However, both studies reported that, increasing the enzyme activity beyond a particular level will not enhance the adsorption capacity. This is because once the saturation level (equilibrium of adsorption) has been reached, it is impossible to

increase the loading on the support. In this study, with increased initial laccase activity, loading was also enhanced. Thus, it has to be hypothesized that the saturation was never reached for any of the three biochars used for the study. As a result, it can be assumed that increasing the activity of laccase in the crude extract will further enhance the adsorption of laccase and thus will increase the immobilization. The aim of this study was to test the loading efficiency/immobilization efficiency of crude laccase on biochars. Nevertheless, increasing the enzyme activity beyond 10 U mL^{-1} through concentration (by ultrafiltration) for the enzymes produced by agro-industrial route was not easily achievable. This was due to the comparatively lower yield of laccase by this method since a maximum of 1 U mL^{-1} of laccase was produced(Lonappan et al., 2017b).

Since, non-purified laccase has been used for the immobilization, competitive adsorption can be expected. Presence of other substances, such as polyphenols, proteins and carboxylic acids (Persic et al., 2017; Sato et al., 2010) in the crude extract can reduce the adsorption of laccase, because the available sites on the biochar can be occupied by these impurities. These factors depend upon the selectivity of the molecules which have to be attached on to the surface of the biochar. This will vary according to the surface features of each biochar. Of the three biochars used in this study, BC-PM had the largest surface area (46.1 m^2 g^{-1}) followed by BC-AS (17.0 m^2 g^{-1}) and BC-PW (14.1 m^2 g^{-1},). This difference in surface area has been reflected in enzyme loading and thus immobilization of enzymes on the biochar surface. BC-PM was found to be most effective (31.4±3.1 U g^{-1}) under optimal conditions followed by BC-AS (24.3±4.8 U g^{-1}) and BC-PW (14.58±3.3 U g^{-1}). The effectiveness of BC-PM can also be directly correlated with their surface features which show the presence of diverse functional groups that can be potential adsorption sites (Lonappan et al., 2017a; Xu et al., 2014).

Previous studies have reported that COOH groups on immobilization supports can enhance the binding of enzymes and laccase in particular on the supports (Cho & Bailey, 1979). The citric acid treated biochars were used for the immobilization laccase and the results are presented as Fig. 4. The selective grafting of COOH groups on biochar surface by citric acid treatment has been reported

in a previous study(Lonappan et al., 2018). However, unexpectedly lowered laccase binding was observed with all the three functionalized biochars.

3.4 Loading efficiency and isotherms

Table 1 presents binding efficiency (%) of the micro-biochars at various laccase concentrations. Laccase concentrations have been presented in international units (IU) and presented for total volume used (10 mL) and 1 g of micro-biochar was used (Cabana et al., 2009). Due to the larger surface area and advanced surface characteristics in comparison with other biochars, BC-PM showed higher binding efficiency that topped at 31.4%. Maximum binding efficiency obtained from BC-AS was 24.3% and with BC-PW, a maximum binding efficiency of 14.58% was achieved. An increasing trend in binding efficiency was observed for all the biochars. For BC-PW, the binding efficiency decreased initially at 25 U and a further increase was observed. However, inconsistencies in results were observed with BC-PW which led to 12% binding efficiency at 5 U and which decreased to 6.4% at 25 U and later an increment to 8.6%, 12.4% and reached a maximum at 14.58% corresponding to 50 U, 75 U and 100 U of laccase activity , respectively. Thus, the immobilization efficiency was barely increased by 2.58% even with 20-fold increase in effective enzyme concentration. This observation can be explained by selective and competitive adsorption and hydrophobic and electrostatic interaction between the laccase and micro-biochar (Lassen & Malmsten, 1996; Nakanishi et al., 2001). Initially, to obtain 5 U enzyme activity, crude extracts were diluted using milliQ water and several fold dilutions (20 folds for 5 U) were made. Thus, this procedure could possibly eliminate the potentially competing adsorbates from the crude laccase and thus effective binding was obtained for laccase. Later, as the enzyme concentration increased, the concentration of other impurities also increased as the dilution reduced. Along with surface area and enzyme concentration, the selectivity of BC-PW for adsorbates is important. In this case, it has to be assumed that BC-PW had a superior affinity towards other adsorbates than the laccase molecule.

For BC-PM and BC-AS, a significant increase in binding efficiency was observed with increasing laccase concentration. This was particularly evident for BC-PM. A consistent increase in binding from 14% to 31.4% was observed from an enzyme concentration (in terms of enzyme activity) of 5 U to 100 U. This phenomenon can be explained based on increased adsorbate (laccase) adsorbent (BC-PM) interactions and thus resulted in higher adsorption (Baysal et al., 2014). Moreover, it must be assumed that in comparison with BC-PW and BC-AS along with an increased surface area, BC-PM exhibited superior affinity towards laccase molecules. However, an increase in binding efficiency from 6% to 24.3% was observed for BC-AS, albeit this phenomenon was not consistent since irregularities in results were observed at 25 U.

Since immobilization process was carried out using adsorption, the adsorption isotherms were plotted to better understand the mechanisms behind protein (laccase binding) to micro-biochars. Langmuir and Freundlich isotherms were employed to study the adsorption process of laccase onto biochars and equation (1) presents Langmuir isotherm model whereas equation (2) presents Freundlich isotherm model.

$$C_e/q_e = 1/Q_0K_L + C_e/Q_0 \quad \text{---------- (1)}$$

$$\log q_e = \log K_F + 1/n \log C_e \quad \text{---------- (2)}$$

Where C_e (U L^{-1}) is the equilibrium concentration of the adsorbate, q_e (U g^{-1}) is the amount of adsorbate adsorbed per unit mass of adsorbent, Q_o and K_L are Langmuir constants for adsorption capacity and rate of adsorption, respectively. K_F is the adsorption capacity of the adsorbent and n is the favorability factor of the adsorption.

Table 2 shows Langmuir and Freundlich adsorption isotherm constants for micro-biochar for laccase adsorption. Langmuir isotherm model was constructed based on the assumption of homogeneous monolayer adsorption onto the surface with no re-adsorption of adsorbate on the surface. Therefore,

for BC-PW laccase adsorption was expected to be homogeneous monolayer adsorption and most likely by physical forces (physisorption) such as electrostatic interactions, H-bonding and Van der Waals forces (dipole-dipole, dipole-induced dipole and London (instantaneous induced dipole-induced dipole) force). However, an R^2 value of 0.92 for Freundlich adsorption isotherm contradicted with this assumption. Even though a 1/n value of 1.052 (isotherm plot not shown) suggested a homogeneous adsorption since adsorption becomes heterogeneous when the slope 1/n equals zero (Baysal et al., 2014). Since this value is greater than 1 even from Freundlich adsorption isotherm constants, homogeneous monolayer adsorption can be expected. Thus, Langmuir isotherm model was suitable for BC-PW. However, for BC-PM and BC-AS, Freundlich adsorption isotherm model was suitable owing to near unity R^2 values (0.96 and 0.98, respectively). Thus, in these two biochars, stronger binding sites on the surface were occupied first and the binding strength decreased with the increasing degree of site occupancy and which reduced the adsorption with time. At the same time, for both these biochars, favorability factor values (1/n) were well above zero and hence instead of heterogeneous adsorption, homogenous adsorption occurred through both physisorption and chemisorption. Of the three biochars, high rate of adsorption (K_L) was shown by BC-AS followed by BC-PM and BC-PW. Thus, theoretically and as per isotherm models, adsorption loading of laccase was faster in BC-AS in comparison with other two micro-biochars.

3.5 Release kinetics (de-immobilization) of immobilized laccase

Results related to desorption been given as Fig. 5. It is evident that a complete desorption can never be expected even under vigorous shaking conditions. However, this particular experiment tried to understand the stability and binding strength of immobilized laccase as well as the nature of release (release kinetics) of various immobilization supports (micro-biochars). Almost all previous studies presented static release (leaching) of enzymes from the support. However, this study presents the desorption/ release of enzymes under mild shaking conditions. It is clear that a very fast release can be expected in this manner due to continuous shaking in comparison with static conditions.

An initial slow release was observed from BC-PM, in comparison with BC-PW and BC-AS even though the initial immobilized enzyme concentration was very high (BC-PM was found to be most effective with 31.4±3.1 U g^{-1} under optimal conditions followed by BC-AS with 24.3±4.8 U g^{-1} and BC-PW with 14.58±3.3 U g^{-1}). This result can be read in line with the isotherm kinetics which suggested possibly higher percentage of chemisorption than physisorption with BC-PM. Presence of metals in BC-PW (Zhang et al., 2013) can enhance the chemisorption that is adsorption by chemical bonds. Comparatively, the faster release was observed from BC-PW and BC-AS which suggested loose binding of laccase on to biochars or physisorption. Thus, a higher percentage of laccase molecules were bound by weak forces, such as Van der Waals forces and electrostatic attractive forces. Moreover, it was observed that at about 10 hours, both BC-AS and BC-PW reached desorption equilibrium despite that the data obtained for BC-PW was not consistent. However, even after 48 h, desorption equilibrium was not reached for BC-PM and which suggested continuous application potential of BC-PM for longer periods. Desorption data was analyzed using kinetics plots and the results are presented in Table 3.

For BC-PM, both pseudo-first-order and pseudo-second-order release kinetics were a good fit which was observed as near unity R^2 value. However, for BC-PW and BC-AS, pseudo-second order release kinetics can be applied to explain the release kinetics. As explained previously, second-order rate constant k_2 confirmed the faster release of laccase from BC-AS and BC-PW in comparison with BC-PM.

3.6 Removal of DCF using laccase immobilized biochars

Biochar obtained with the best binding of laccase was used for the removal studies of diclofenac. Batch mode experiments were carried out with each enzyme immobilized micro-biochar samples and the results are shown in Fig. 6.

The contribution of adsorptive (Lonappan et al., 2017a) as well as enzymatic removal (Lonappan et al., 2017b) over time has already been reported for the degradation DCF for each biochar as well as

for laccase. In these studies, similar experimental conditions were used. With all samples, near 100% removal was observed over time. As enzymes are immobilized onto biochars, continuous adsorption and biotransformation of DCF can be expected. It can be hypothesized that, a series of continuous adsorption of DCF onto the vacant sites (sites available after immobilization of laccase) of micro biochar and simultaneous degradation of DCF through laccase can be expected. DCF is often considered as a compound resistant to laccase degradation (Nguyen et al., 2014; Yang et al., 2013). Poor oxidative efficiency of laccase and steric hindrance between laccase and the target molecule are the major reasons for inadequate degradation of certain micro-pollutants (d'Acunzo & Galli, 2003). Simultaneous and complete removal of DCF is possibly due to the enhancement of electron transfer between laccase and DCF after adsorption on the GAC surface. Furthermore, in line with previous release kinetics experiments (section 3.6), the immobilized laccase can be used for several cycles without loss in enzyme activity.

3.7 Storage and stability and shelf-life of immobilized laccase

Low thermal and pH stability (narrow pH range in activity) and loss of catalytic activity of enzymes after one cycle and consumption loss while using in water environment have remained as major obstacles in large-scale environmental remediation applications (Jesionowski et al., 2014). Thus, past studies investigated the improvement of enzymes stability through immobilization on solid supports (Cowan & Fernandez-Lafuente, 2011; Jesionowski et al., 2014; Mateo et al., 2007; Sheldon & van Pelt, 2013). About 80% reduction in laccase activity (residual) was observed for free-form laccase while being stored at 4 °C over 60 days. The loss in residual activity was about 38% after 20 days and 65% after 40 days. However, higher enzyme activity stability was observed with all immobilized biochar samples at 4± 1 °C over 60 days. The loss in residual activity was less than 35% for all laccase immobilized biochar samples over 60 days. After 60 days, laccase immobilized BC-PM lost 21% activity, laccase immobilized BC-PW lost 24% residual activity and laccase immobilized BC-AS lost 32% residual activity. A similar but lower storage stability was observed for laccase immobilized on chitosan/poly (vinyl alcohol) composite nanofibrous membranes (Xu et al.,

2013), however, this study employed covalent binding. Another study reported up to 98% retention in residual activity of immobilized laccase while immobilized on Eupergit supports (Lloret et al., 2012).

To the best of our knowledge, for the first time, this study presented concise and first hand information on adsorptive immobilization of crude enzyme (laccase) on various biochars by using the principles of physical chemistry (adsorption). Moreover, this study demonstrated one potential application of laccase immobilized micro-biochar for the removal of an emerging contaminant DCF. Thus, the laccase immobilized micro-biochar can be used for several other applications including but not limited to soil remediation, wastewater treatment etc. and which further signifies the importance of this study.

4. Conclusions

Adsorptive immobilization of crude laccase on pine wood (BC-PW), pig manure (BC-PM) and almond shell (BC-AS) micro-biochars were investigated using the principles of physical chemistry and through isotherms. With all biochars, as the initial activity of laccase increases in the crude solution, the binding capacity also increased and thus the immobilization efficiency. BC-PM was found to be most effective (31.4±3.1 U/g) at 10 U $mL^{-1}$of enzyme activity followed by BC-AS (24.3±4.8 U/g) and BC-PW (14.58±3.3 U/g). Homogeneous monolayer adsorption was key mechanism behind enzyme binding on biochar. Immobilized laccase exhibited superior storage ability/ shelf life over free laccase and which was quantified to be more than 3 times. Immobilized laccase degraded diclofenac with near 100% and obtained within 5 hours at an environmentally relevant concentration (500 ug L^{-1}).

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Table 1: Immobilization efficiency of laccase at various activities on micro-biochars

Micro-biochar	Theoretical binding efficiency of laccase (%) at				
	5 U	**25 U**	**50 U**	**75 U**	**100 U**
BC-PW	12	6.4	8.6	12.4	14.58
BC-PM	14	16.4	16.6	18.53	31.4
BC-AS	6	2	9	22.4	24.3

BC-PW: pine wood biochar, BC-PM: pig manure biochar, BC-AS: almond shell biochar

Table 2: Langmuir and Freundlich adsorption isotherm constants for micro-biochar for laccase adsorption

Micro-biochar	Langmuir isotherm model			Freundlich isotherm model		
	Q_0 (U g^{-1})	K_L (LU^{-1})	R^2	K_F (U g^{-1})	n	R^2
BC-PW	6.87	8.28×10^{-5}	0.96	1288.2	0.95	0.92
BC-PM	21.4	7.26×10^{-5}	0.74	2917.4	0.80	0.96
BC-AS	2.54	1.20×10^{-4}	0.33	28543	0.648	0.98

BC-PW: pine wood biochar, BC-PM: pig manure biochar, BC-AS: almond shell biochar

Table 3: Desorption kinetics of laccase from biochars

Micro-biochar	Pseudo-first order model			Pseudo-second order model		
	q_e, cal. (U g^{-1})	k_1 (h^{-1})	R^2	q_e, cal. (U g^{-1})	K_2 (g $U^{-1}h^{-1}$)	R^2
BC-PW	----	-----	0.1663	16.0	1.95×10^{-4}	0.99
BC-PM	-891.2	0.00092	0.99	2500	5.42×10^{-8}	0.99
BC-AS	------	------	0.369	30.3	8.12×10^{-2}	0.99

BC-PW: pine wood biochar, BC-PM: pig manure biochar, BC-AS: almond shell biochar

q_e and q_t are desorbed (U g^{-1}) laccase from biochar at equilibrium and at time t (hour) respectively and k_1 is the rate constant of desorption (h^{-1}). k_2 (g U^{-1} h^{-1}) is the desorption rate constant of pseudo-second-order desorption.

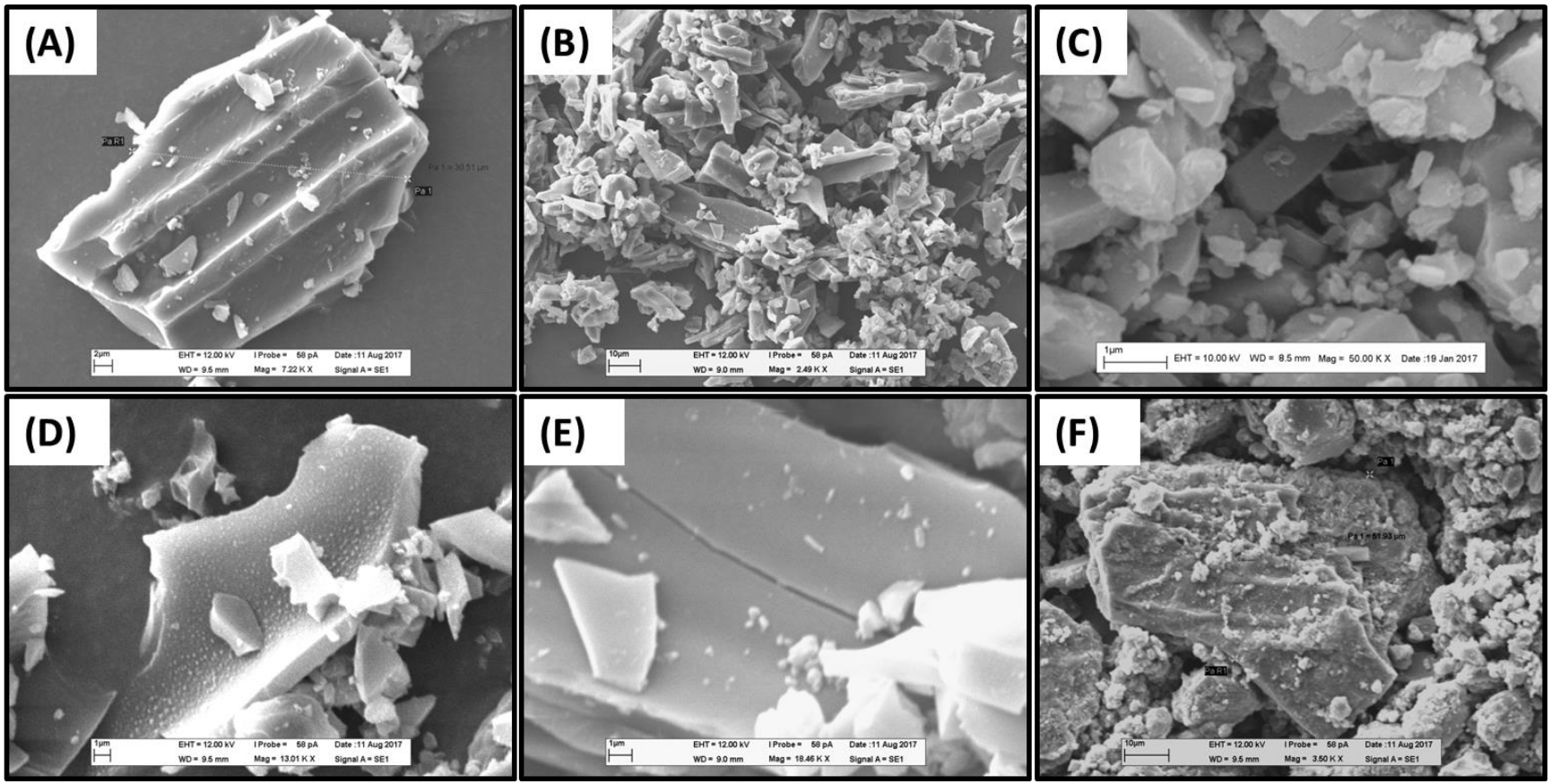

Figure 1: SEM of micro-biochars before and after laccase immobilization (A, D: pine wood biochar before and after laccase immobilization; B, E: pig manure biochar before and after laccase immobilization; C, F: almond shell biochar before and after laccase immobilization)

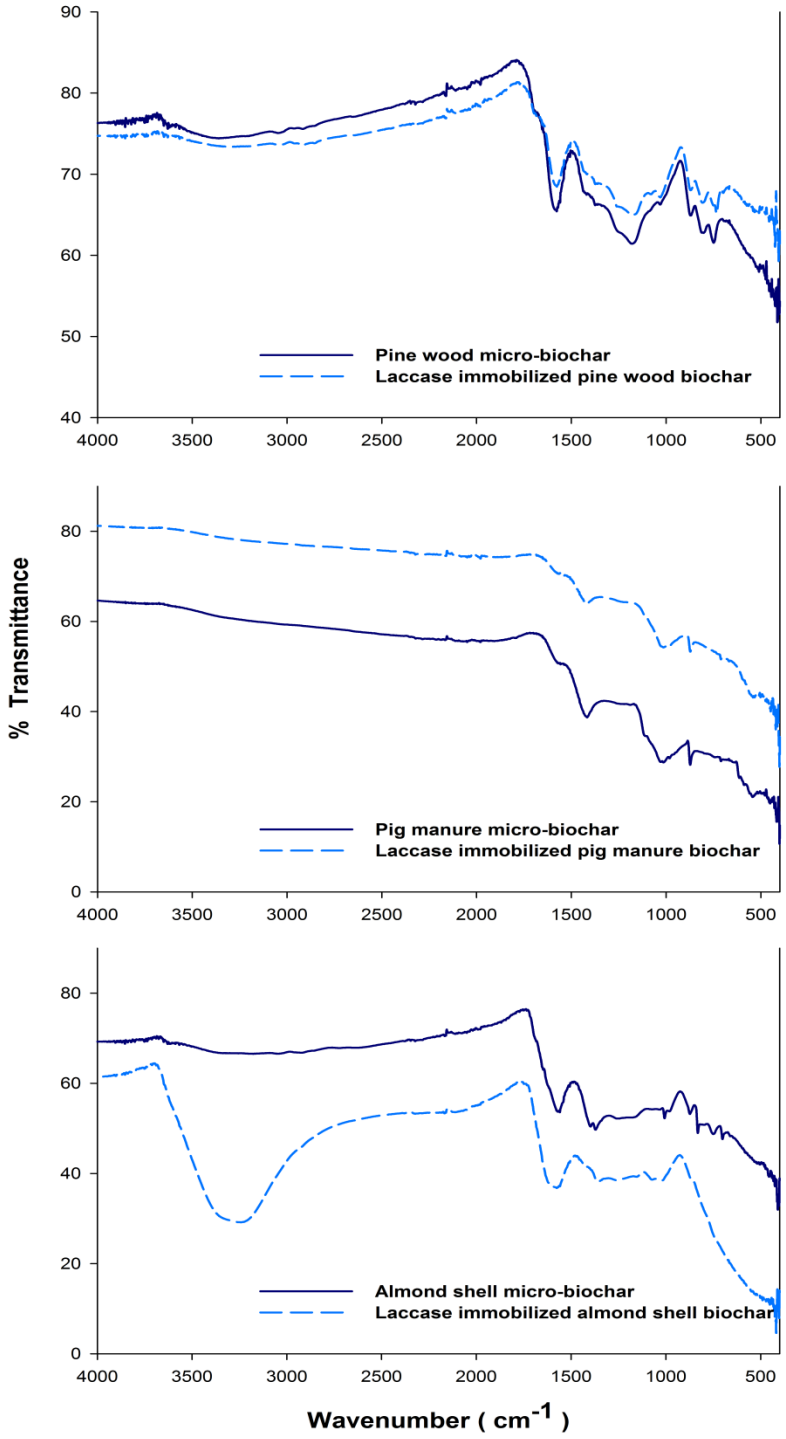

Figure 2: FTIR spectra of micro-biochars before and after laccase immobilization

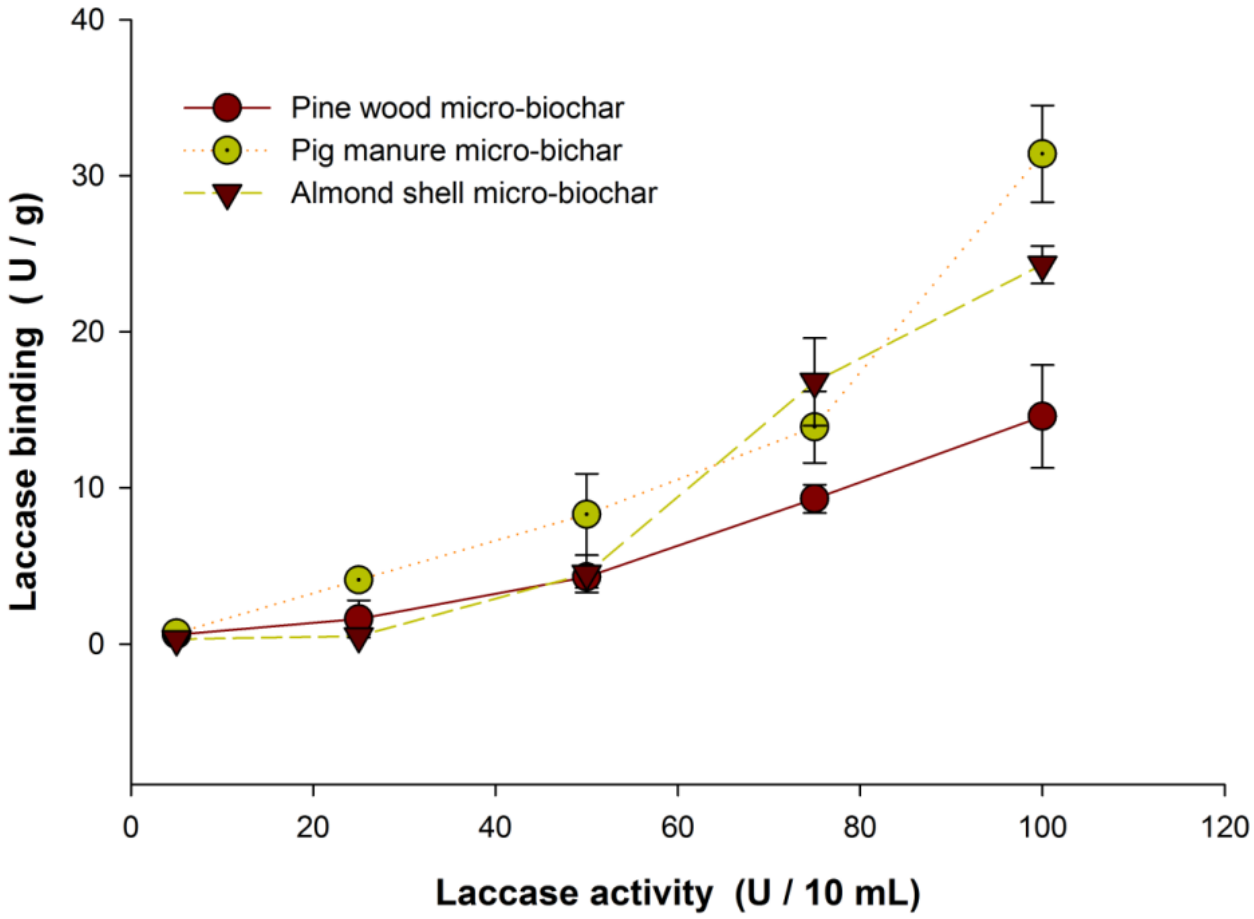

Figure 3: Laccase loading on non-functionalized biochars at different laccase activities

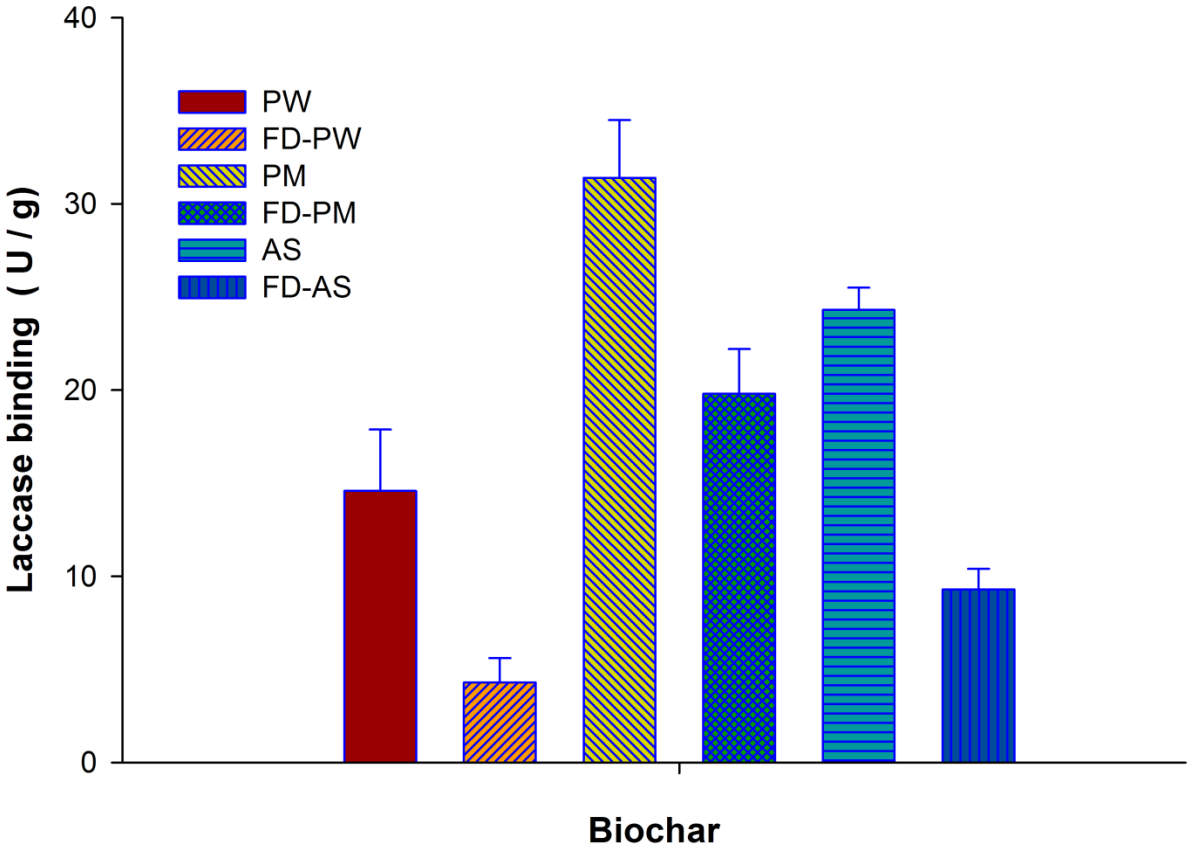

Figure 4: Laccase loading on functionalized biochars at 10 U mL^{-1} laccase activity

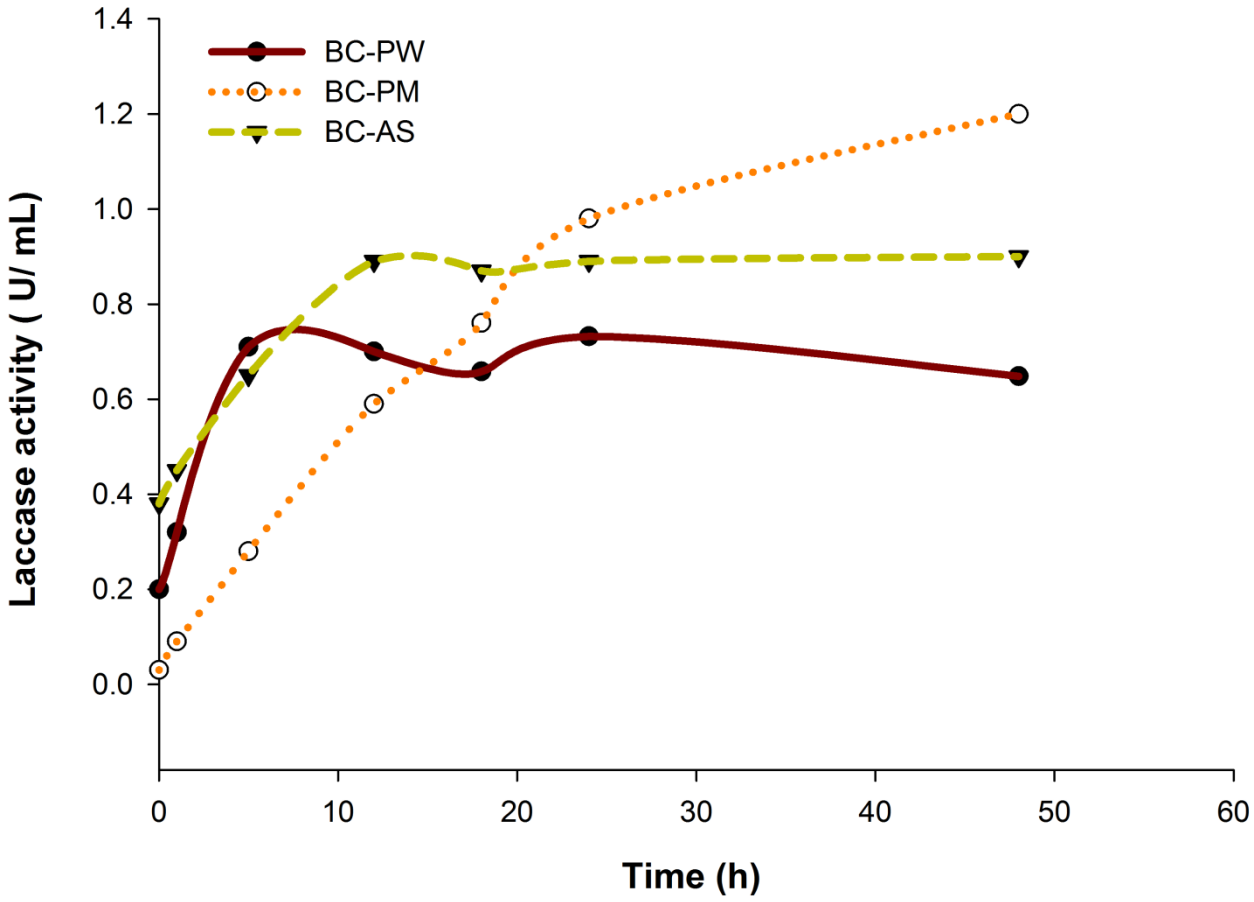

Figure 5: Release (de-immobilization) of immobilized laccase on micro-biochars

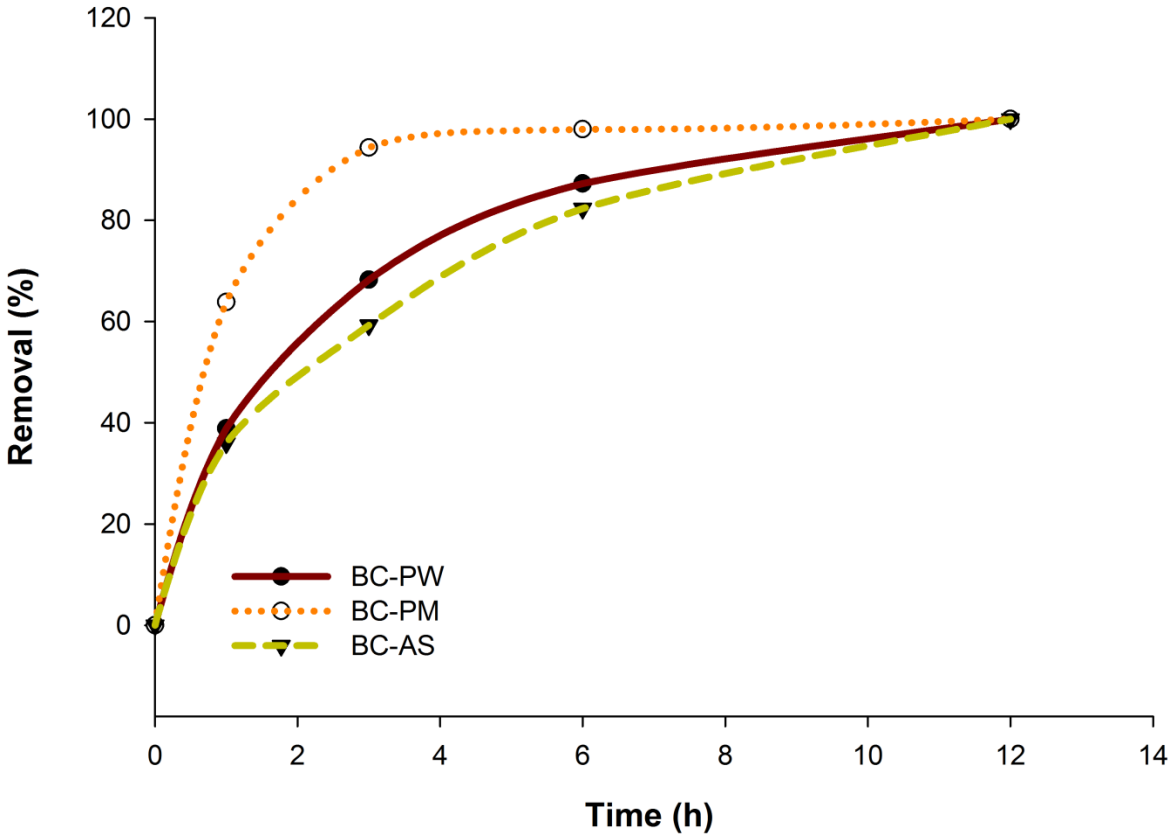

Figure 6: Removal of DCF using enzyme immobilized micro-biochar over time

PART 3

COVALENT IMMOBILIZATION OF LACCASE ON CITRIC ACID FUNCTIONALIZED MICRO-BIOCHARS DERIVED FROM DIFFERENT FEEDSTOCKS AND REMOVAL OF DICLOFENAC

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WATER RESEARCH (UNDER REVIEW)

RÉSUMÉ

La remédiation enzymatique des micropolluants peut être une méthode efficace et durable, cependant, les préoccupations de stabilité des enzymes représentent un inconvénient pour cette approche. L'immobilisation des enzymes sur un support solide peut améliorer la stabilité ainsi que les propriétés catalytiques des enzymes. Dans cette étude, le biochar dérivé de diverse matière première a été utilisé comme support d'immobilisation tout en prenant en compte les propriétés carbones négatives et durables du biochar. La laccase brute partiellement purifiée (concentrée) a été immobilisée par covalence sur des microbiochars de pin (BC-PW), de fumier de porc (BC-PM) et de coquille d'amande (BC-AS) en utilisant une glutaraldéhyde optimisée à 5% p/v après une série d'optimisations. De plus, le prétraitement à l'acide citrique a amélioré la capacité de liaison à la laccase de tous les microbiochars et la liaison à la laccase la plus élevée de 40,2 ± 1,8 U g^{-1} a été observée avec BC-PM (34,1 ± 1,1 U g^{-1}). La liaison accrue de la laccase sur les BC-PM par rapport aux biochars dérivés du bois a été attribuée à la plus grande surface (46,1 m^2 g^{-1}) de BC-PM. BC-PW a montré une meilleure stabilité au pH, à la chaleur, au stockage et au fonctionnement, comparativement aux BC-AS et BC-PM. Lors de l'application du microbiochar lié à la laccase, une élimination complète a été observée en 2 heures en mode discontinu avec 0,5 g de BC-PM lié à la laccase à une concentration environnementale de 500 µg L^{-1} dans l'effluent d'eaux usées. Environ 40% de l'activité de la laccase a été retenue avec tous les microbiochars liés à la laccase après 5 cycles de traitement au diclofénac.

Mots-clés: Immobilisation covalente; Biochar; Laccase; Acide citrique, stabilité des enzymes; Diclofénac

ABSTRACT

Enzymatic remediation of micropollutants can be an efficient and sustainable method, however, stability concerns regarding enzymes is a drawback for this approach. Immobilization of enzymes on the solid support can improve the stability as well as catalytic properties of enzymes. In this study, biochar derived from various feedstocks were used as immobilization support while considering biochars carbon negative as well sustainable properties. Partially purified (concentrated) crude laccase was covalently immobilized onto pine wood (BC-PW), pig manure (BC-PM) and almond shell (BC-AS) micro-biochars using optimized 5 % w/v glutaraldehyde after series of optimizations. Moreover, citric acid pretreatment improved the laccase binding capacity of all the micro-biochars and the highest laccase binding of 40.2±1.8 U g-1 was observed with BC-PM while compared with raw BC-PM (34.1±1.1 U g-1) The enhanced binding of laccase on BC-PM over wood derived biochars was attributed towards the higher surface area (46.1 m2 g-1) of BC-PM. BC-PW showed better pH, thermal, storage and operational stability, compared with BC-AS and BC-PM. . On applying the laccase bound micro-biochar, complete removal was observed in 2 hours under batch mode with 0.5 g of laccase bound BC-PM at an environmentally relevant concentration of 500 µg L-1 in wastewater effluent. About 40 % of the laccase activity was retained with all the laccase-bound micro-biochars after 5 cycles of diclofenac treatment.

Keywords: Covalent immobilization; Biochar; Laccase; Citric acid, Enzymes stability; Diclofenac

1. Introduction

Enzymatic remediation/bio catalysis is gaining attention in the recent past particularly, for the bioremediation of recalcitrant compounds. Ligninolytic enzymes are being used for the past several decades and can be a potential solution for environmental remediation, such as for the bioremediation of micro-pollutants (Durán and Esposito 2000). Laccases are the most prominent enzymes in this class along with lignin peroxidase and manganese peroxidase (Shraddha et al., 2011). Laccases are used in industrial wastewater treatment, food industry (juice and wine clarification), decolourization of dyes and for the delignification of woody fiber (Fernandez-Fernandez et al., 2013, Homaei et al., 2013, Vernekar 2009), and most importantly for degradation of recalcitrant pollutants (Majeau et al., 2010b). Cost-effective and eco-friendly production of laccase is a prerequisite for industrial process viability and previous studies demonstrated waste/residue-based production of laccase (Lonappan et al., 2017a, Postemsky et al., 2017, Sharma et al., 2017).

Even if enzymes can be produced by economically convenient methods, application of free enzymes for the bioremediation of recalcitrant pollutants is not industrially feasible. Immobilizing the enzymes on a solid support is a prerequisite as it can increase its activity, stability and most importantly, its reusability (Brena et al., 2013, Zucca and Sanjust 2014). The characteristics of an ideal support for enzyme immobilization include inexpensive nature; able to load a significant amount of enzyme per unit weight; low hydrophobicity; inertness after immobilization; microbial resistance; pH, thermal and mechanical resistance (Zucca and Sanjust 2014). Several immobilization supports have been previously studied (Brena et al., 2013). However, application of biochar as an immobilization support is not thoroughly studied nor well established. Often biochar is produced from waste/residual materials and thus it has the potential for both cost-effectiveness as well as waste valorization. Moreover, biochar shows strong physicochemical resistance on top of its high sorption ability and capacity to exchange electrons (Naghdi et al., 2017, Prevoteau et al., 2016). Laccase immobilization on pine wood nano-biochar and its mineral acid functionalized derivatives was previously studied (Naghdi et al., 2017) and ascertained significant improvement in storage and stability. However,

biochars produced from different feedstock and under varied conditions exhibit completely different properties (Lonappan et al., 2018a, Sun et al., 2014, Zhao et al., 2013). Thus, further studies are required to establish the potential applicability of biochar as an enzyme immobilization support. In general, two types of immobilization approaches are used and they are (i) adsorption (adsorptive immobilization) and; (ii) chemical bonds formed between the support and enzymes (covalent immobilization). Covalent immobilization of enzymes results in slow release of enzymes and better storage ability/shelf-life. Thus, covalent immobilization of enzymes is preferred for long-lasting, real-scale and continuous applications. Further, in this study, micro-biochars produced from various feedstocks under different conditions were used for the immobilization of crude laccase which fills lacunae existing in this research. To the best of our knowledge, biochar feedstock effects and surface chemistry were not previously studied for covalent laccase immobilization through covalent binding on various micro-biochars. This study investigates the possibility of covalent immobilization of laccase onto micro-biochars prepared from the feedstock, such as pine wood (BC-PW), pig manure (BC-PM) and almond shell (BC-AS). Surface modified derivatives of biochars using citric acid were also tested for immobilization of laccase because of possible grafting of -COOH groups.

Diclofenac (DCF) is a largely sold and extensively used drug with an estimated global consumption of 1443 ± 58 tons per year (Acuña et al., 2015). The toxicity and environmental effects of DCF have been studied previously and studies revealed that DCF residues in the environment can toxic towards both terrestrial and aquatic animals (Lonappan et al., 2016a, Petrie et al., 2015, Vieno and Sillanpää 2014). For instance, Mehinto et al reported that even concentrations such as 1 μg L^{-1} can have significant effects on liver, gills, and kidney of rainbow trout (*Oncorhynchus mykiss*) (Mehinto et al., 2010). Due to these significant toxic concerns and comparatively high consumption as well detection rate in wastewater, DCF was selected as the model contaminant to test the efficiency of laccase immobilized micro biochar system which was developed in this study.

2. Materials and Methods

2.1 Materials

Pinewood and almond shell biochar samples were gifted by Pyrovac Inc. (Quebec, Canada). Pine white wood (80% v/v) spruce and fir (20% v/v) was pyrolyzed at 525±1°C under atmospheric pressure to produce pine wood biochar. Almond shell biochar was produced from the almond shell at 520±1°C for 4 h. Pig manure biochar was procured from Research and Development Institute for Agri-Environment (IRDA), Quebec, Canada and produced from a solid fraction of pig slurry by pyrolysis at 400±1 °C for 2 h. All biochars were ground using 'Retsch RS 200' vibratory disc mill at 1000 rpm for 10 min and obtained particles were sieved through ASTM 200 numbered sieve to obtain a uniform particle size of less than 75 µm. Moreover, surface treatment of micro-biochars was carried out using 2 M citric acid by shaking biochar with citric acid for 24 hours. A detailed description of surface modification was given in a previous publication (submitted-under review) (Lonappan et al., 2018b).

Apple pomace was used as the substrate for laccase production and was procured from Vergers Paul Jodoin Inc, Quebec. *Trametes versicolor* (ATCC 20869) was selected as fungi for the production of laccase. Detailed production procedure of laccase has been presented elsewhere (Lonappan et al., 2017a). In short, about 25 g of apple pomace was used as the substrate and mycelial layer from one Petri-plate was used as the inoculum. Fermentation was carried out for 9 days at 30±1°C with 75±1% moisture. On the 9^{th} day, laccase from fermented broth was extracted using sodium phosphate buffer (pH 6.5, (10/1 v/w). The supernatant obtained after centrifugation was filtered using a rough filter paper and later filtered through ultrafiltration in a Sartorius stedim − SartoJet ultrafiltration system (Göttingen, Germany). Sartocon slice hydrosart Cassettes (10 kDa and 100 kDa) were used as the filtration membranes. Filtrates collected between 10-100 kDa were harvested and subjected to enzyme assay. After concentration, laccase activity of 10 U mL^{-1} (10,000 U L^{-1}) was obtained and was used for the immobilization.

Glutaraldehyde and diclofenac sodium salt (98%; CAS 15307-79-6) was purchased from Fisher Scientific (Ottawa, ON, Canada) and internal standard (IS), diclofenac-d_4 was obtained from C/D/N isotopes Inc. (Montreal, QC, Canada). The molecular weight of diclofenac is 296.1 g mol^{-1}, log K_{ow} is 4.51 and pK_a is 4.15. All other chemicals used in this study were of analytical grade and was obtained from Fisher Scientific or Sigma-Aldrich (Canada).

2.2 Enzyme assay

Laccase activity was spectrophotometrically measured at 420 nm using 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Laccase oxidizes ABTS into ABTS radical ($ABTS^+$) and is blue in color. The assay solution was prepared using 100 mM citrate-phosphate as the buffer (pH 4.5), 0.5 mM ABTS and enzyme at an extinction coefficient (ε) of 36,000 M^{-1} cm^{-1} were used for the calculations. Crude laccase - ABTS mixture was incubated for about five minutes and the average per minute increase in absorbance was calculated from the slope of the straight line which was obtained while plotting absorbance versus time. One unit of laccase activity (U mL^{-1}) is defined as the production of 1 μM ABTS cation per minute under assay conditions (Bourbonnais and Paice 1992, Collins and Dobson 1997a).

2.3 Covalent immobilization of concentrated crude laccase on micro-biochars

2.3.1 Surface modified micro-biochars

Initially, micro-biochars BC-PW, BC-PM, and BC-AS were tested as the solid support for immobilization. Later, organic acid functionalized derivatives of the biochars were used for the immobilization to study for any enhancement in immobilization/loading of laccase through functionalization. Citric acid was used for the functionalization of micro-biochars following a previous study which demonstrated that these functionalization methods are effective for introducing –COOH functional groups on micro-biochars (Lonappan et al., 2018b). The optimal concentration of glutaraldehyde was added to surface functionalized micro-biochars and incubated under shaking for

5 h and later crude laccase was added to this mixture and incubated for another 3 hours. After 3 h of incubation, the biochar was removed, and the enzyme activity was measured.

2.3.2 Covalent immobilization of laccase onto micro-biochars

A constant solid: liquid ratio of 1:10 of biochar was used throughout the study (about 100 U of laccase per gram). Crude concentrated laccase, glutaraldehyde , and biochar were mixed using a vortex blender (Fisher Scientific 02215370 Vortex mixer-deluxe 120V) at 1200 rpm and at room temperature; 25 °C and pH 6.5 .

Three strategies were tested for the effective immobilization of laccase onto biochars. In the first method, 10 mL of crude laccase (10,000 U L^{-1} activity) was mixed with 1g of biochar for 5 h followed by the addition of glutaraldehyde and mixing for 12 h (at room temperature, 25 °C) with intermediate sampling every 3 h. This was to ensure the initial adsorption of laccase on to micro-biochars and later reinforcement by cross-linking. Glutaraldehyde concentrations of 1 %, 2.5 %, 5% and 10 % (*v/v* in the final volume) were tested along with this approach to optimize its concentration. In the second approach, glutaraldehyde (optimized concentration) was mixed with micro-biochar for 5 h to activate the immobilization sites; either amine groups or carboxylic acid groups which followed by the addition of crude laccase and shaking for another 3 h (at room temperature, 25 °C). In the third approach, crude laccase, cross-linking agent and micro-biochar was mixed together overnight (for about 12 h).

After immobilization, enzyme- biochar mixture was centrifuged, and the liquid portion was decanted which was further analyzed for the remaining laccase activity. The solid fraction was washed twice using deionized water, centrifuged and the liquid used for washing was discarded. Enzyme-loaded biochar was dried under compressed air by maintaining a temperature lower than 25±1°C. Later, laccase immobilized biochar was completely dried at room temperature and stored at 4°C.

To ensure the immobilizationand further release of laccase onto biochars, 0.1 g of laccase-immobilized biochar was shaken with 1 mL of sodium phosphate buffer at pH 6.5 for 15 minutes. This

mixture was centrifuged in a tabletop centrifuge and enzyme activity in the supernatant was analyzed.

2.4 Stability, release and reusability of laccase immobilized on micro-biochars

The pH, temperature and storage stability of crude and immobilized laccase on biochars were studied. Crude enzyme mixture containing laccase was used as the control/blank for all these experiments. The pH stability was tested for pHs of 2.5, 4.5, 6.5, 8.5 and 10.5. About 0.1 g of immobilized laccase samples was added to 5 mL buffer solutions having the above-mentioned pH. The release of laccase was studied at 25 °C while shaking in vortex blender (Fisher Scientific 02215370 Vortex mixer-deluxe 120V) at 750 rpm for 24 hours. Laccase activity in the supernatants was measured after centrifugation at the 24^{th} hour. Similarly, to study the thermal stability, the samples were incubated at temperatures ranging from 10°C to 70°C for 12 h at pH 6.5. Enzyme activity measurement was carried out at the respective temperatures. The storage stability/shelf-life of the crude and immobilized laccase samples was measured by enzyme activity of the immobilized laccase over a period of 5 weeks by measuring the residual activity once in a week. Laccase immobilized samples were left at room temperature while studying the storage stability.

2.5 Characterization of micro-biochars with covalently bound laccase

Representative samples were selected based on laccase loading and used for the characterization. The surface morphology of the laccase immobilized biochars was investigated using EVO® 50 smart scanning electron microscope (SEM) (Zeiss, Germany). Before analysis, the samples were coated with gold using a SPI-module sputter coater to minimize charging effect. Specific surface areas of micro-biochars before and after immobilization were measured using Brunauer, Emmett, and Teller (BET) technique (Autosorb-1, Quantachrome, USA). Moreover, Fourier transform infrared spectra of micro-biochars and laccase immobilized micro-biochars were recorded using a Nicole IS50 FT-IR Spectrometer (Thermo Scientific, USA). Transmittance was measured at 4 cm^{-1} resolution with 16 scans per sample.

2.6 Diclofenac degradation experiments

Laccase immobilized biochars with maximum laccase loading were selected from each biochar class (BC-PW, BC-PM, and BC-AS) and was tested for the degradation of DCF. Degradation experiments were carried out at room temperature and in wastewater (effluent collected from Quebec Urban Community wastewater treatment plant) having a pH of 6.35. DCF was spiked to the wastewater and the initial concentration of DCF in wastewater was maintained at 500 µg L^{-1} and which was considered as environmentally relevant (Lonappan et al., 2016c). The general composition of wastewater from Quebec Urban Community wastewater treatment plant has been presented elsewhere (Naghdi et al., 2017, Puicharla et al., 2014). About 0.5 g of laccase immobilized micro-biochar was incubated with 25 mL DCF solution at 25 °C for 6 hours with continuous sampling in every 30 minutes. Supernatant from samples (by collecting about 1mL each and centrifuging in a tabletop centrifuge) was collected and mixed with equal amount of methanol (purity >99.8%, Fisher Scientific Ottawa, ON, Canada) to determine enzyme activity. Samples were stored at 4°C, until analysis. Quantification of DCF was carried out using LDTD-APCI-MS/MS method and detailed procedure is described elsewhere (Lonappan et al., 2016b).

Reusability of laccase immobilized micro-biochars was studied by measuring the residual activity of laccase in laccase-immobilized biochars over 5 cycles of diclofenac degradation. Diclofenac degradation experiments were carried out in batches as described before. In this experiment, in the first batch, about 1.2 g of biochar and 120 mL of DCF solution was used. After each cycle (6 hours), the samples were washed using milli-Q water, centrifuged, and the recovered biochar was dried using compressed air and reused. However, 100% recovery of biochar was not obtained after washing. The amount of biochar added after each cycle was reduced in each batch by maintaining the same solid: liquid ratio as the first batch.

2.7 Quality control

All experiments were carried out in duplicates. Means and standard deviations are calculated and data is presented with ±SD. Except for immobilization experiments, one-way ANOVA was carried out with 95% confidence level using Sigmaplot software (version 11.0, Systat Software Inc.) and the results which had $p < 0.05$ were considered as significant.

3. Results and discussion

3.1 Characterization of micro-biochars covalently bound with laccase

Scanning electron micrographs of micro-biochar as well as enzyme loaded biochar (selected laccase immobilized samples from BC-PW, BC-PM and BC-AS (untreated) are given as Figure 1 A, B, C, D, E and F. Figures A and B represent BC-PW before and after laccase immobilization, respectively. Similarly, C and D represent BC-PM and E and F represent BC-AS. A clear and visible change in the surface texture was not observed after immobilization of laccase. However, clumping was observed after laccase immobilization with all the micro-biochars and this observation was more prominent with BC-AS as shown in Fig. 1(F). This phenomenon can be attributed to the changes in surface charges by the addition of immobilizing agents as well as the addition of crude laccase. The electron micrographs showed the representative surface and porous texture of micro-biochars. Moreover, the molecular weight of laccases is between 60–90 kDa (Shraddha et al., 2011) and which approximately corresponds to a particle size of less than 5 nm (Erickson 2009). The micrographs showed a minimum magnification up to 1µm and further magnification was not possible due to instrument limitations. Thus, the electron micrographs serve the sole purpose of presenting the surface texture of micro-biochar. However, another study presented encapsulated laccases on polyvinyl alcohol/chitosan and showed diameters between 1-3 µm for the enzyme capsules (Xu et al., 2015).

Fourier-transform infrared spectra of micro-biochars and laccase immobilized biochars (raw biochar not treated with citric acid) are presented in figure 2. Broad peaks which were observed around 3300

cm^{-1} were attributed towards the presence of primary, secondary amine and amides and which can be correlated to the presence of laccases as the molecular formula of laccase show the presence of several NH groups. However, as regards to the laccase in pure or impure form, only limited information is available for FT-IR interpretation (Nitta et al., 2002, Ragusa et al., 2002).

Micro-biochars were having surface areas of 14.1 $m^2 g^{-1}$ (BC-PW), 46.1 $m^2 g^{-1}$ (BC-PM) and 17 $m^2 g^{-1}$ (BC-AS) respectively. After covalent immobilization surface area has been decreased to 4.85 $m^2 g^{-1}$ (BC-PW), 38.8 $m^2 g^{-1}$ (BC-PM) and 12.6 $m^2 g^{-1}$ (BC-AS). The reduction in surface area was because the crude enzyme (proteins) and glutaraldehyde was occupied on the surface along with the possible agglomeration of micro-biochar since cross-linking agent was used.

3.2 Covalent immobilization of laccase on untreated micro-biochars

3.2.1 Optimization of glutaraldehyde concentration and incubation time

The bifunctional versatile nature of glutaraldehyde permits its application to almost any surface for the immobilization of proteins. Glutaraldehyde is capable of reacting with the amine groups at the surface of both enzyme and supports through the formation of Schiff's bases and Michael's adducts (Isabelle Migneault 2004). In this study, as an initial optimization, 1 %, 2.5%, 5% and 10 % (*v/v,* in the final volume) glutaraldehyde was used for the immobilization of laccase on BC-PW, BC-PW, and BC-AS. Results revealed that application of 5% glutaraldehyde resulted in the optimal loading/binding of laccase onto biochars (data not shown) in comparison with the other glutaraldehyde concentrations. Moreover, enzyme activity/loading decreased with 10% glutaraldehyde concentration on prolonged contact with glutaraldehyde, such as for 12 hours (overnight) and this observation was unanimous with all the three biochars (BC-PW : 9.8 %; BC-PM: 11.1%; BC-AS : 12.4%). This observation can be ascribed to the further interaction of laccase with a high concentration of glutaraldehyde, which can cause inactivation of laccase (Pezzella et al., 2014). A blank experiment without the addition of biochar was conducted by mixing only glutaraldehyde and crude laccase and which further confirmed the inactivation of laccase by a higher concentration of

glutaraldehyde on prolonged contact. On the other hand, lower glutaraldehyde concentrations, such as 1% resulted in a better immobilization on prolonged contact with glutaraldehyde for 12 h. However, this increase was not comparable with the laccase loading observed at 5% glutaraldehyde for 3 hours of incubation. Moreover, very high concentrations of glutaraldehyde can induce glutaraldehyde-glutaraldehyde interactions rather than laccase-glutaraldehyde interactions, since glutaraldehyde is a bifunctional reagent. Thus, 5% glutaraldehyde and 3 hours of contact after the initial adsorption step was ascertained to be optimal and further studies were conducted in these conditions. In a similar study about laccase immobilization on silica, Tavares et al. (2013) also observed that 5 % glutaraldehyde was optimal for the covalent immobilization of laccase (Tavares et al., 2013). Optimal glutaraldehyde concentration of 5% was used also for laccase immobilization on the nano-copper incorporated electro spun fibrous membrane (Xu et al., 2017). Another study on laccase immobilization on perlite revealed that 1-5 % glutaraldehyde and incubation for 4 hours optimal for effective immobilization of laccase (Pezzella et al., 2014).

3.2.2 Effect of various methods and micro-biochars

Three approaches were used for the immobilization of laccase onto micro-biochars as described in section 2.3.2. In the first approach, micro-biochars were mixed with crude laccase for about 5 hours. This facilitated the adsorption of laccase on the micro-biochars and after 5 hours 1% (*v/v*) glutaraldehyde was added, the reaction was allowed to continue overnight (12 h, with intermittent sampling). This procedure allowed glutaraldehyde to cross-link both the laccase and the support which is micro-biochar (Betancor et al., 2006).The above-mentioned optimization (section 3.2.1) was carried out using this method (addition of glutaraldehyde in the second step: method 1). Excellent binding of laccase onto biochars was observed as illustrated in Fig. 2 A maximum of 16.18±0.3 U g^{-1} laccase was covalently bound to BC-PW whereas 34.1±1.1 U g^{-1} and 25.3±2.8 U g^{-1} laccase binding were observed with BC-PM and BC-AS, respectively. In the second approach, micro-biochars were initially shaken with 5 % (*v/v* in the final mixture) glutaraldehyde for 5 hours and crude laccase was added to this mixture and continued incubating for another 4 hours (addition of glutaraldehyde in the

first step: method 2). In this approach, immobilization is expected to occur through ionic exchange between the biochar (which was pre-activated by glutaraldehyde) and laccase (Betancor et al., 2006). Results obtained are shown in Fig.3. In method 2, almost identical results with ±5 % difference in enzyme binding were observed in comparison with method 1. Highest laccase binding was observed with BC-PM (33.4±2.2 U g^{-1}) followed by BC-AS (26.7±1.7 U g^{-1}) and BC-PW (15.9±0.9 U g^{-1}). In the third approach, crude laccase, cross-linking agent and micro-biochar was mixed together overnight (for about 12 hours- method 3). However, in comparison with method 1 and method 2, significant reduction in laccase binding was observed as shown in Fig.3. Laccase binding with all biochars as was reduced up to 31%. For instance, with BC-PM, only 23.4±3.2 U g^{-1} laccase binding was observed instead of 34.1±1.1 U g^{-1} laccase binding with method 1. Two possible reasons can be ascertained towards reduction in enzyme activity/binding onto micro-biochars. Firstly, the possible denaturation of laccase by glutaraldehyde on prolonged incubation (Pezzella et al., 2014) for about 12 hours instead of 4 hours with the other two methods. The second possible reason can be overcrowding of surface with enzymes and multipoint attachment of enzymes onto the biochar carriers (Bayramoğlu et al., 2008). Methods 1 and 2 were found to be effective and didn't showed any significant ($p < 0.05$) difference loading capacities. As a result method-1 was selected as the optimized method for further studies (supplementary data - univariate tests for significance of loading capacity).

As shown in Figures 1 and 2, biochars completely exhibited different surface texture, morphology, surface chemistry and functional groups. This was attributed to the feedstock selection and pyrolytic conditions (Sun et al., 2014, Zhao et al., 2013). Biochars generally (with few exceptions) exhibit a basic pH (Li et al., 2013) and all the three biochars used in this study showed pH values ranging from 8 to 10.5. However, on the contrary, most of the biochars exhibits considerably high cation exchange capacity (CEC) (Singh et al., 2010) and thus showed cationic nature. Low-ionic strength and the cationic nature of the surface allowed a rapid immobilization of protein with the help of glutaraldehyde (Betancor et al., 2006). Of the three biochars studied, BC-PM was found to be the best support for laccase immobilization in comparison with BC-PW and BC-AS. Manure

derived biochars, such as BC-PM showed much higher CEC cation exchange capacity in comparison with wood-based biochar similar to BC-PW and BC-AS (Singh et al., 2010). Thus, high CEC can enhance the cationic nature of biochar which can further increase the enzyme binding through covalent binding with glutaraldehyde. Moreover, the abundant presence of various surface functional groups including aldehyde, amine carboxylic acids and hydroxyl groups on the biochar surface ((Lonappan et al., 2018a) as presented in Fig.2 are capable of forming bonds with glutaraldehyde and which can enhance the loading. Moreover, higher binding of laccase on BC-PM can be attributed toward the high surface area of BC-PM in comparison with BC-PW and BC-AS.

3.3 Citric acid functionalized micro-biochars and glutaraldehyde

To improve laccase binding on micro-biochars, micro-biochars were initially treated with citric acid. Citric acid being an organic acid eliminates the toxic concerns regarding the application of mineral acids which is very popular for surface modification of biochars and preparation of activated carbon (Azargohar and Dalai 2006). After citric acid functionalization, the carboxylic acid functional groups grafted on biochar surface can be observed as shown in Fig.4 (strong and broad peak around 3000 cm^{-1}). Improved laccase binding on biochars was observed in comparison with raw untreated micro-biochar and the results are presented as Fig.5. Similar order in laccase binding was observed as in section 3.2.2; BC-PM (40.2±1.8 U g^{-1}) having the highest laccase binding followed by BC-AS (31.8±1.3 U g^{-1}) and BC-PW (20.10±3.6 U g^{-1}) and which corresponded to a theoretical loading efficiency of 40.2 %, 31.8 % and 20.1 %, respectively. In comparison with non-functionalized micro-biochar and under similar conditions (method 1), about 17 % improvement in laccase binding was observed with BC-PM, 16 % increase was observed with BC-AS and 20% improvement was observed with BC-PW. Similar improvement in laccase binding was observed with mineral acid treated activated carbon and biochar and the authors attributed the increase towards the enhanced available surface area and removal of debris on the surface of the support (Naghdi et al., 2017, Nguyen et al., 2016). In addition, unlike mineral acids, the action of organic acids could be more delicate which will improve the functional groups on the surface of biochar than making a big change

in the surface area as represented in Fig. 4. Thus, along with improved surface area, involvement of other factors, such as chemical reactions involved in covalent binding of laccase cannot be overruled. Initial, rapid adsorption of laccase onto biochar surface was hypothesized due to the expected improved surface area and the presence of –COOH functional groups that can enhance the adsorption of laccase (Nguyen et al., 2016). Later, the remaining laccase undergoes cross-linking through the addition of glutaraldehyde (Betancor et al., 2006) and this cross-linked laccase was further linked to the biochar surface with glutaraldehyde. In a related study, citric acid was used for the cross-linking of hydroxyl propyl methyl cellulose (HPMC) with the help of NaH_2PO_4catalyst and which improved the water vapor barrier by about 34% (Coma et al., 2003).

3.4 Stability of immobilized laccase

3.4.1 Effect of pH and temperature on immobilized laccase

Due to the changes in the ionization state of amino acids with pH (Haki and Rakshit 2003, Nitta et al., 2002), changes in pH can affect any enzyme including laccase. However, enzymes are highly stable in immobilized form and are susceptible to pH and temperature changes in the immobilized form (Brena et al., 2013, Fernandez-Fernandez et al., 2013). The pH effect of free and immobilized laccase was studied from pH of 2 to 10 and the results are presented as Fig.5 (A). In the immobilized form, highest laccase activity was observed at pH 4.5 and which was higher than the activity observed at pH 2.5 for all biochars. However, this difference was not very high (less than 10% difference). It has to be noted that laccase activity is measured at pH 4.5 since which was found to be the optimal pH for highest laccase activity under standard test conditions (Bourbonnais and Paice 1992, Collins and Dobson 1997b). There was a rapid reduction in activity was observed unanimously for all biochars along with pH 8.5 and 10.5. However, at pH 2.5 and 6.5 laccase activities were found to be almost similar and just below the activity which was observed at 4.5. Similar observations were previously reported that is optimal pH of 4 for immobilized laccase on nano-pine wood biochar; in porous glass beads (Leonowicz et al., 1988) and pH 5 for immobilized laccase on polyacrylonitrile

(PAN) beads (Catapane et al., 2013). In the present study, for all the biochars, the optimal pH was found to be 4.5. Laccase in free form is not stable under high acidic and basic conditions and significant changes in laccase activity can be observed (Lonappan et al., 2017a, Naghdi et al., 2017). From Fig. 6(A), it is evident that immobilization has significantly improved stability of laccase and provided resistance to inactivation with pH changes. This enhanced spectrum of pH ranges extended the laccase applicability to large number of pollutants having high acidic and basic pH's. At a pH of 6.5 (approximates pH of wastewater), BC-PM immobilized laccase has maintained 77% of its activity while comparing with pH 4.5 which has shown highest activity. Under the same conditions, BC-PW maintained 86% activity and BC-AS maintained 83% activity. The lowest laccase activity was observed at pH 10.5 for all the biochars. However, even at pH 10.5 while comparing with pH 4.5, BC-AS maintained 61% residual activity followed by BC-PW (60%) and BC-PM (50%). Thus, maximum loss of 50 % was observed even with a highly basic pH and the immobilization significantly improved the pH stability of the laccase. Moreover, it is interesting to note that the immobilized laccase on wood derived biochar (BC-PW, BC-AS) exhibited higher stability than laccase immobilized on manure-derived biochar (BC-PM) as shown in fig 7.

Figure 6 (B) illustrates the changes in laccase activity along with temperature. Highest laccase activity was observed at 40 °C unanimously for all biochars. At 10°C and 25°C, similar laccase activity was observed. However, after 40°C, a steady decrease in laccase activity bound biochars was observed. A similar observation of highest activities between 40-50 °C was observed for the degradation of octylphenol through immobilized laccase on PAN beads (Catapane et al., 2013) and immobilized laccase on porous glass beads (Leonowicz et al., 1988). Another study reported that activity of the immobilized laccase was better conserved at 40 °C than the free form (Cabana et al., 2007). In this study, all the other results were compared with the highest activity observed at 40°C. About 61 % activity was maintained at 70°C for BC-PW, while 59.7% was maintained for BC-PM and 72.6% for BC-AS. Thus, overall less than 40% loss in residual activity was observed even at 70°C. Moreover, almost similar (±5% change) residual activity was observed at 55°C and 70°C. The enhanced stability and resistance to denaturation is an important property of immobilized enzymes

on solid supports as observed in several previous studies (Bayramoğlu et al., 2008, Brena et al., 2013, Cabana et al., 2007, Fernandez-Fernandez et al., 2013, Haki and Rakshit 2003, Leonowicz et al., 1988, Nguyen et al., 2016). Enhanced thermal stability can be due to increased substrate diffusion at higher temperatures (Xu et al., 2013). Moreover, while studying the effect of biochar feedstock, it was observed that similar to pH resistance, the immobilized laccase on wood derived biochar (BC-PW, BC-AS) exhibited higher thermal stability than laccase immobilized on manure-derived biochar (BC-PM).

As a result, it can be concluded that immobilization on micro-biochars confers to the enzyme higher resistance to pH and thermal inactivation, and feedstock selection of biochar was an important influencing factor for pH and thermal stability of immobilized enzymes.

3.4.2 Storage stability

Laccases in free form are not stable under normal storage conditions, such as at room temperature or under refrigeration and they lose enzyme activity gradually over time (Haki and Rakshit 2003, Majeau et al., 2010a). This is due to the denaturation of proteins (structural and conformational changes) over time and immobilization of enzymes on the solid support can reduce this effect (Leonowicz et al., 1988). Figure 6 (C) presents the changes in residual laccase activity immobilized on various micro-biochars at 25±1 °C (room temperature) over a period of five weeks. It was observed that after 5 weeks, BC- PW has maintained 80% of its residual activity followed by 74 % for BC-AS. However, BC-PM has maintained only 65.6% of its initial activity after 5 weeks. These results are similar to the results which were observed with pH and temperature effect on stability. Thus, it can be ascertained that wood derived biochars exhibit much more protection while preventing denaturation of laccase activity in comparison with manure-derived biochars. The improved stability of laccase can be attributed to the improved resistance capacity of enzymes towards the conformational changes and lower flexibility of the laccase in immobilized form (Leonowicz et al., 1988). In previous similar studies, a similar improvement in stability was observed and the percent of improvement varied between studies (30-70%) (Pezzella et al., 2014, Tavares et al., 2013) and the

nature of immobilization support and the extent and mechanism of binding. Moreover, laccase immobilized on PAN membranes has reported up to 90% retention of laccase activity in 3 weeks (Xu et al., 2013).

3.5 Application of laccase immobilized micro-biochars for removal of DCF

3.5.1 Removal of DCF

Figure 7 (A) illustrates the removal of DCF by various laccase immobilized micro-biochars. With all the three micro-biochars, near 100 % removal was observed within few hours. With BC-PM, near 100 % removal was observed in 2 h (98.9%) and which can be attributed to the high laccase binding with BC-PM Moreover, BC-PM had a better surface area in comparison with the other two micro-biochars used in this study and it is already established that BC-PM showed excellent adsorption capacity towards DCF (Lonappan et al., 2018a). With BC-PW treated wastewater, DCF was not detected from 3rd hour onwards. Near 100 % removal was observed with BC-AS after 4 hours of treatment (98.9%) and in the following hours, DCF was not detected in the wastewater. Similarly, almost 100 % removal of DCF was observed with BC-PW after 5 hours of treatment. The removal efficiency of DCF followed exactly the same order as enzyme bound to the micro-biochars and thus the release and availability of more laccase and better availability of surface area for adsorption can be correlated with these observations in changes with time of degradation or rate of degradation. It can be hypothesized that some portion of diclofenac was adsorbed to the biochar surface and may not have undergone degradation even though 100 % removal was observed. However, laccase bound to biochar retained its activity even after several cycles of DCF degradation experiments (explained in section 3.6.2). Thus, the adsorbed diclofenac will eventually be degraded due to the presence of laccase on biochar surface in the simultaneous process. In the previous study, where laccase was immobilized to granular activated carbon (GAC) applied for the degradation of DCF in column mode, 100% removal of DCF was observed in 24 hours (Nguyen et al., 2016). However, this experiment was in column mode and which limits the contact time that was observed at the extended time for

degradation. Moreover, a very high concentration of DCF was used in this study and which was 2.5 mg L^{-1}. In another study where 12.5 mg L^{-1} of DCF was used as the initial concentration, complete removal of DCF was observed within 6 hours using laccase immobilized onto multi-walled carbon nanotubes (Xu et al., 2015). Previously under almost identical conditions, DCF adsorption onto micro-biochars (Lonappan et al., 2018a) and DCF degradation using free laccase (Lonappan et al., 2017b) were studied and both of these studies reported more than/ near about 5 hours for the complete removal of DCF. However, with laccase immobilized BC-PM, this has been reduced to almost 2 hours in this study. Steric hindrance and/ or blockades in the transfer of electrons between laccase and DCF can be the potential reason for the lower removal rate of DCF or prolonged time for the removal of DCF as reported in the previous study on laccase degradation. However, immobilization of laccase on solid support could have enhanced the electron transfer between laccase and DCF after immobilization since adsorption of DCF on micro-biochar can enhance effective contact between laccase and micro-biochars (Nguyen et al., 2016).

3.5.2 Reusability/ operational stability of covalently immobilized laccase

The operational stability/ reusability of micro biochar bound laccase was investigated for 5 cycles of batch DCF degradation experiments and the results are presented as Fig. 7 (B). BC-PW bound laccase retained 46 % activity after 5 cycles as BC-AS bound laccase retained 43 % activity after 5 cycles. BC-PM was found to be the best support for laccase immobilization out of the three biochars. In this study, BC-PM retained 40 % of laccase activity after 5 cycles. A similarretention of laccase activity (35%) was reported for granular activated carbon bound laccase in a previous study (Nguyen et al., 2016). Previously laccase immobilized on multiwalled carbon nanotubes retained about 73 % activity after 7 cycles of ABTS oxidation (Xu et al., 2015). However, in the same study, under similar conditions, only 48% retention in laccase activity was observed with polyvinyl alcohol (PVA)/chitosan (CS) bound laccase. In this study also, as illustrated in Figure 7 (B) variations in retention of laccase activity was observed with different immobilization support. As a result, it can be ascribed that, operation stability depended upon the immobilization support physicochemical properties, binding

strength between laccase and the support and leaching kinetics of laccase from the support and which can vary significantly.

This study presented a method for the covalent immobilization of crude on various micro-biochars. In addition, this study demonstrated one potential application of covalently bound laccase on micro-biochar for the removal of an emerging contaminant DCF. Covalently bound laccase demonstrated superior storage and operation stability and thus can be used for several environmental applications including but not limited to soil remediation, wastewater treatment etc.

4. Conclusions

Partially purified (concentrated) crude laccase was covalently immobilized onto BC-PW, BC-PM, and BC-AS using 5 % glutaraldehyde. BC-PM (34.1±1.1 U g^{-1}) showed the highest laccase binding capacity followed by BC-AS (25.3±2.8 U g^{-1}) and BC-PW (16.18±0.3 U g^{-1}). Citric acid pretreatment improved the laccase binding capacity of all the micro-biochars and showed up to 20 % improvement in laccase binding with BC-AS. The enhanced surface area and CEC of manure-based biochars over wood derived biochar was attributed to the better binding of laccase on BC-PM. However, wood-derived biochars (BC-PW and BC-AS) showed better pH, thermal, storage and operational stability over manure-derived biochars (BC-PM). Laccase immobilized micro-biochars were used for the removal of micro-pollutant, diclofenac and complete removal of diclofenac was observed in 2 hours under batch mode with 0.5 g of laccase bound BC-PM at an environmentally relevant concentration of 500 µg L^{-1} in the wastewater effluent. Moreover, more than 40 % of the laccase activity was retained with all the laccase-bound biochar after 5 cycles of diclofenac treatment in which each cycle lasted for 6 hours.

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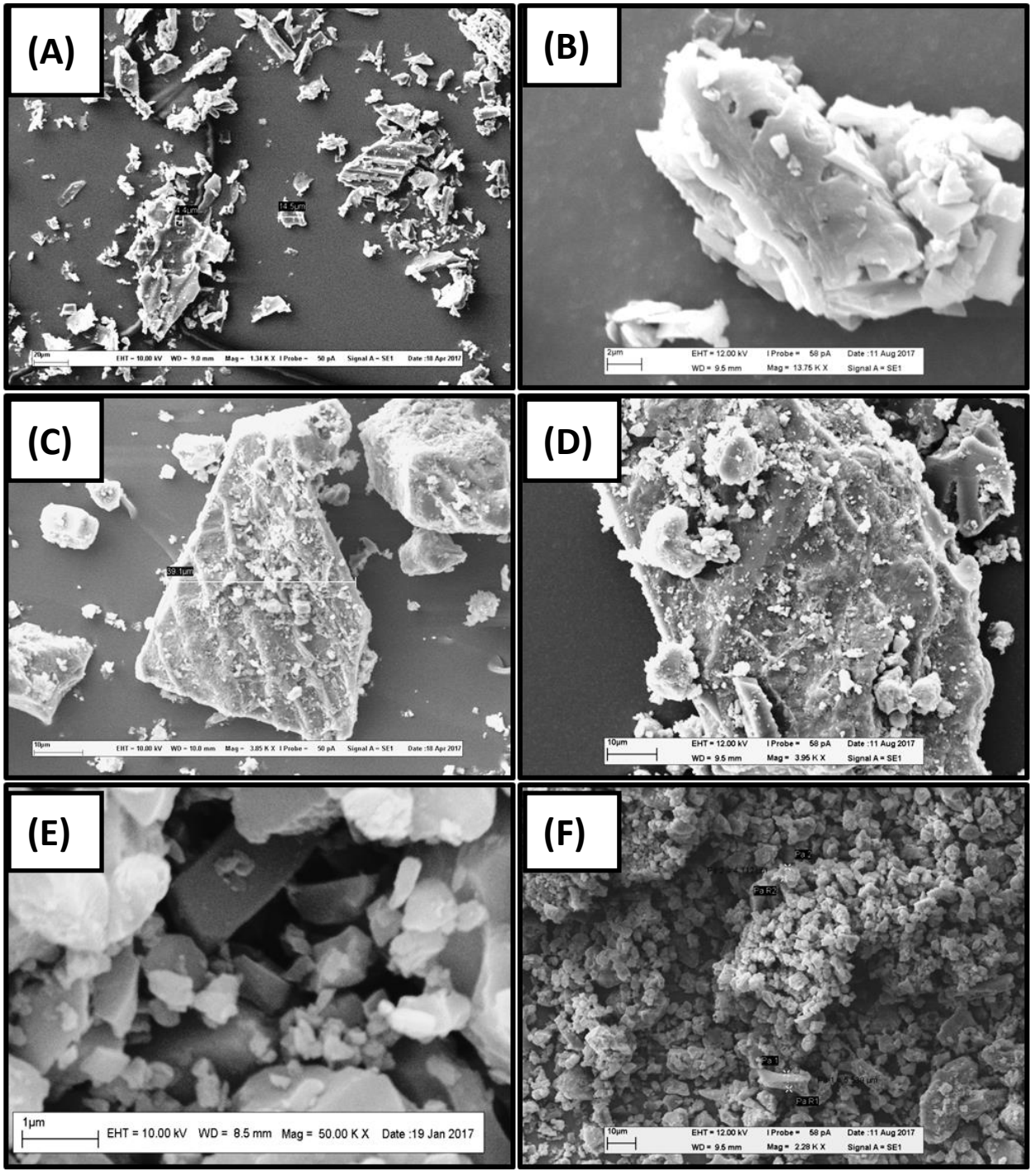


Figure 1: Scanning electron micrographs of biochars before (A (pinewood biochar), C (pig manure biochar), E (almond shell biochar)) and after (B, D, and F respectively) laccase immobilization

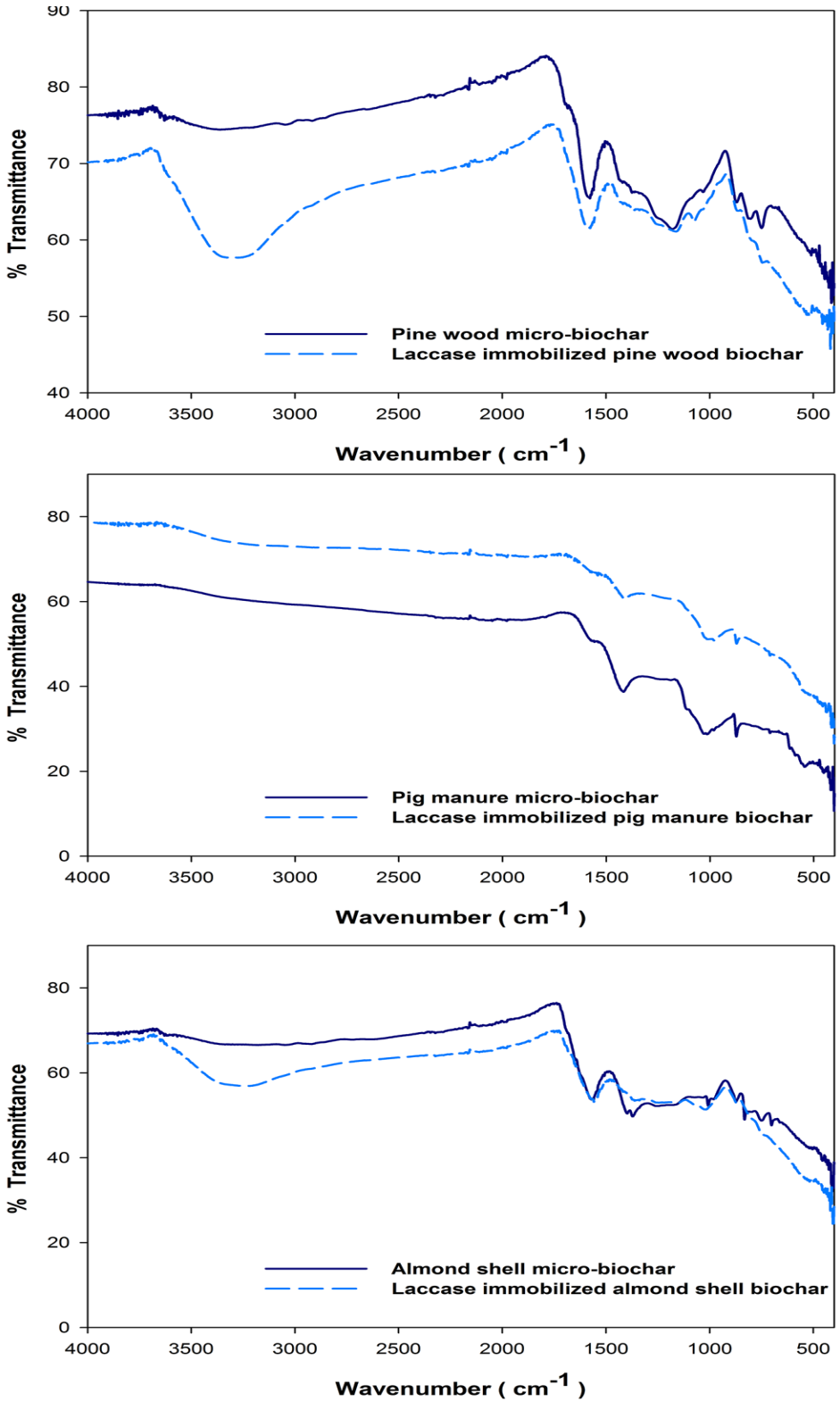


Figure 2: FTIR spectra of micro-biochars before and after laccase immobilization

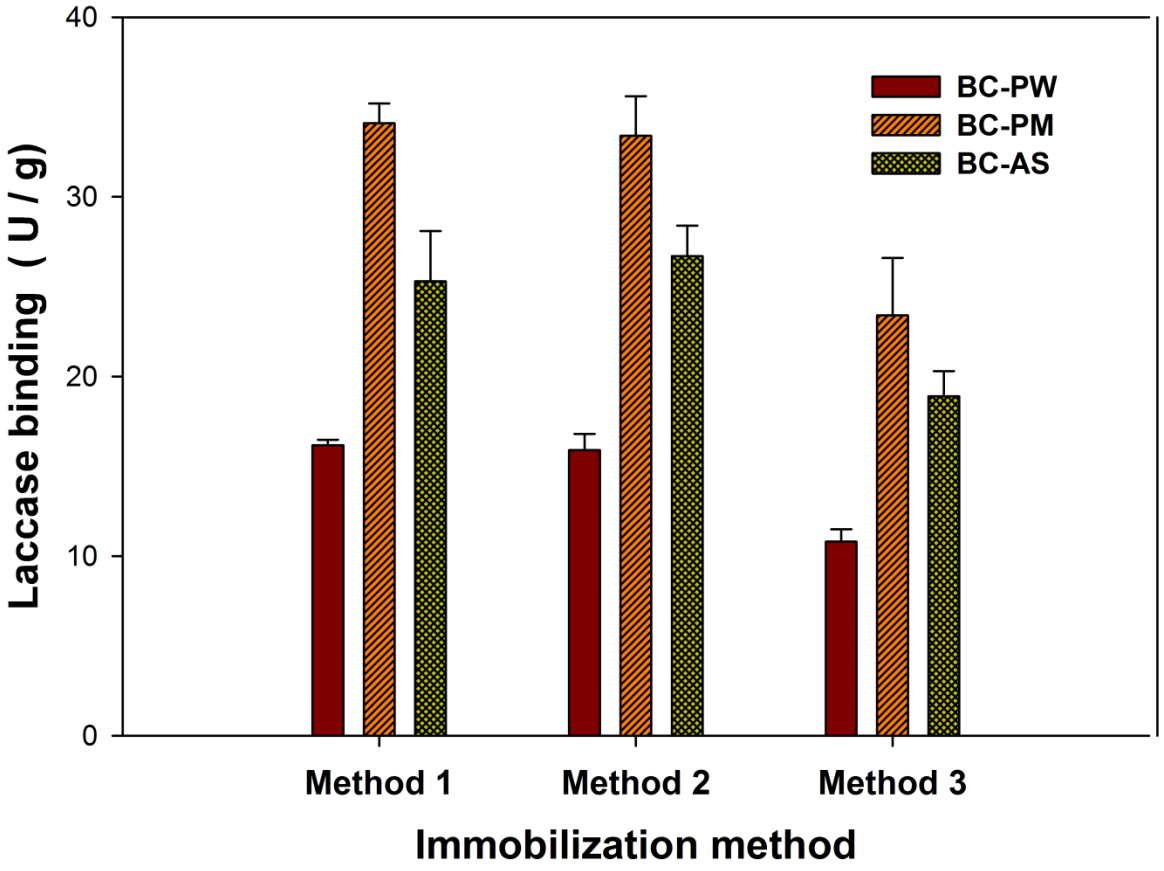


Figure 3: Covalent-immobilization of laccase onto micro-biochars by different methods

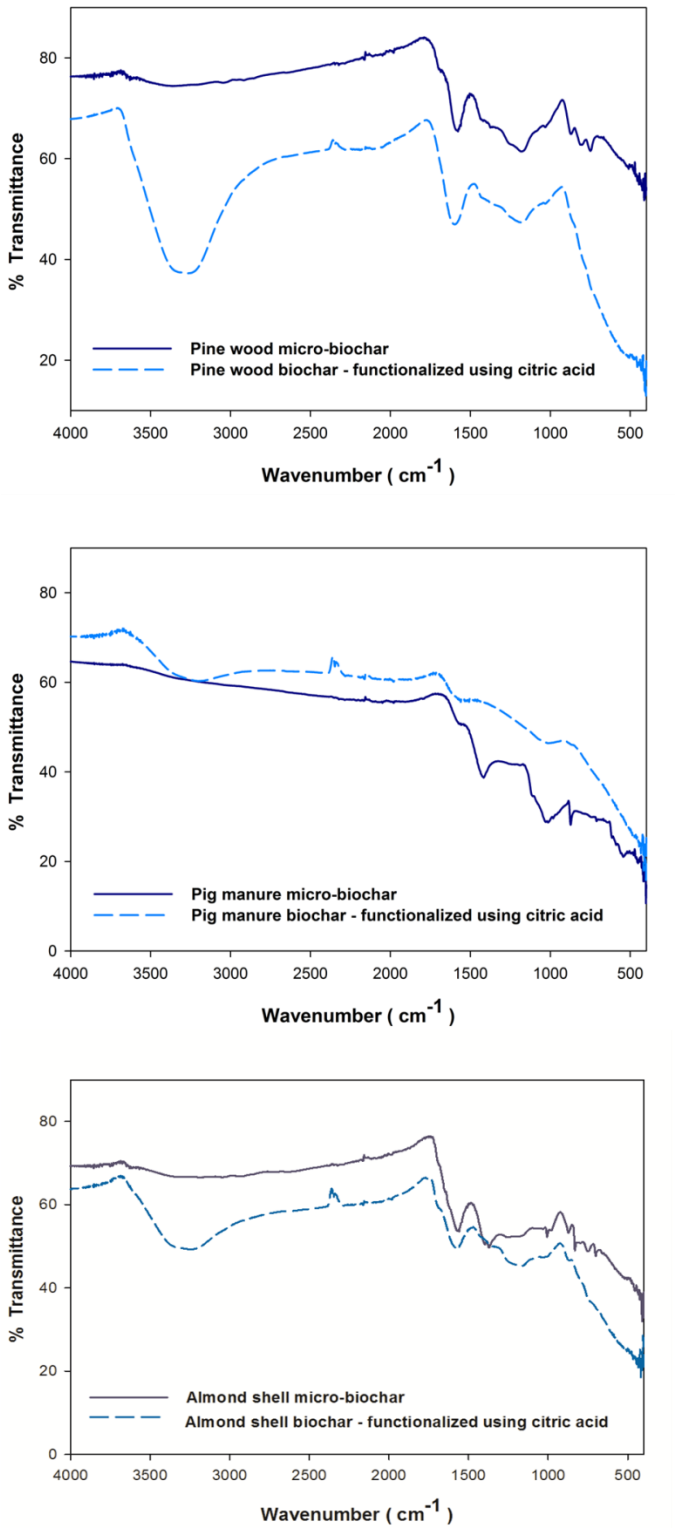
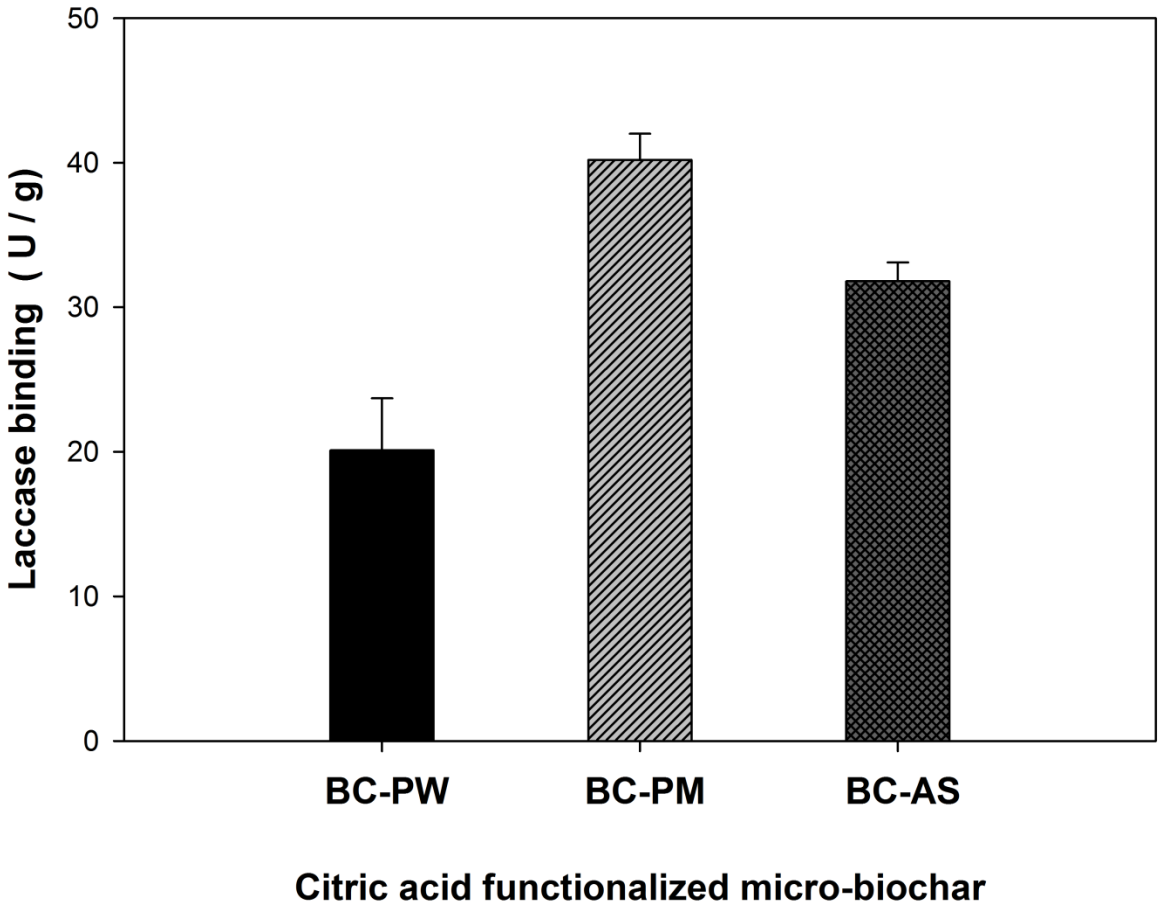
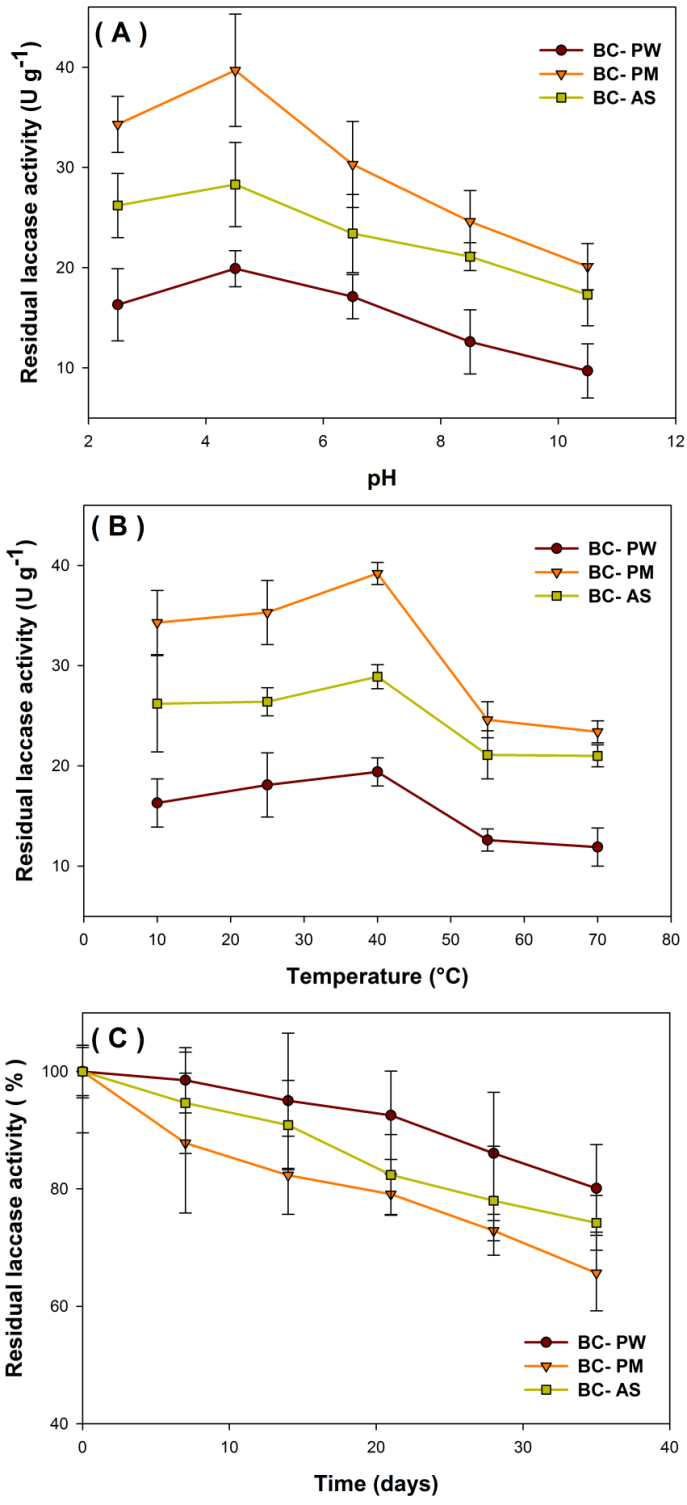


Figure 4: FTIR spectra of citric acid functionalized micro-biochars



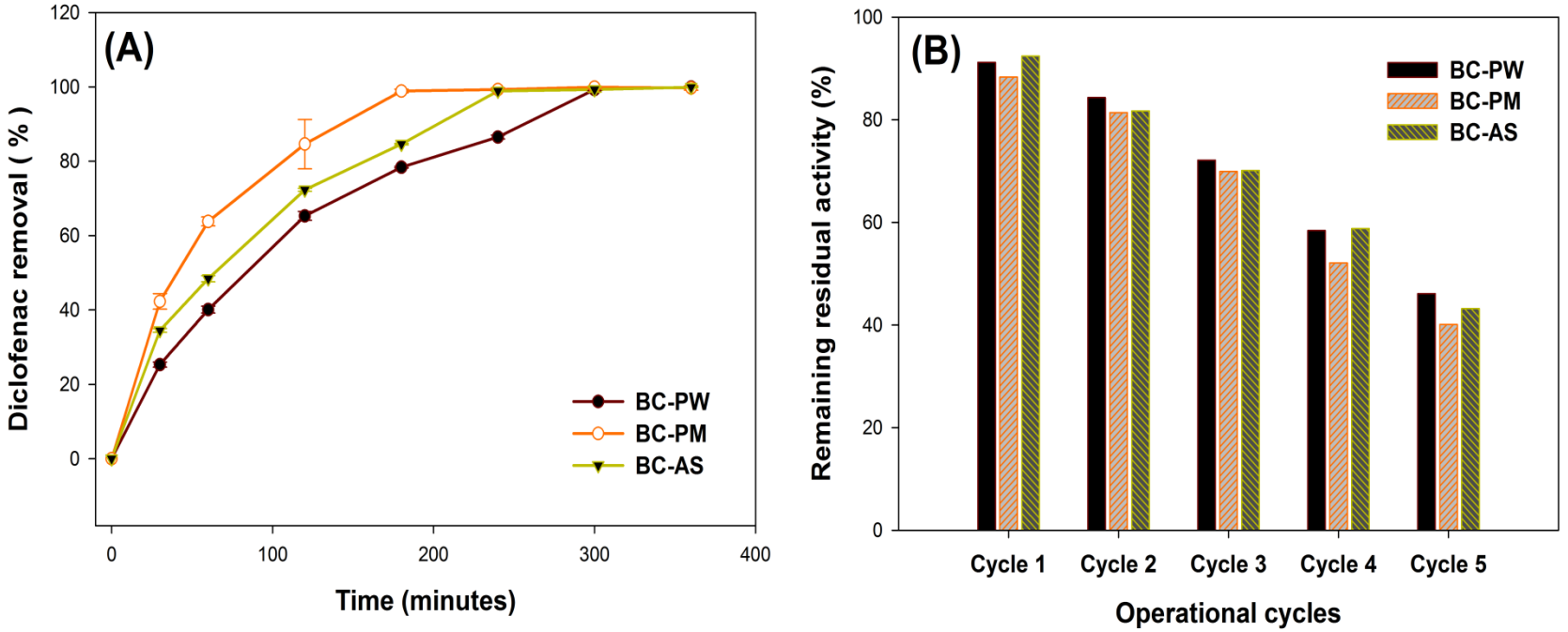
BC-PW: pine wood biochar, BC-PM: pig manure biochar, BC-AS: almond shell biochar

Figure 5: Covalent-immobilization of laccase onto citric acid functionalized micro-biochars (method-1)



BC-PW: pine wood biochar, BC-PM: pig manure biochar, BC-AS: almond shell biochar

Figure 6: Effect of A) pH; B) temperature and; C) 5 weeks storage on activity of covalently immobilized laccase on citric acid functionalized micro-biochars



BC-PW: pine wood biochar, BC-PM: pig manure biochar, BC-AS: almond shell biochar

Figure 7: (A) Diclofenac removal by laccase immobilized micro-biochars and; (B) operational stability of laccase immobilized micro-biochars

CHAPTER 5

THE BIOCHAR- ENZYME IMPREGNATED MICROSYSTEM (BEMS)

REMOVAL OF DICLOFENAC USING MICROBIOCHAR FIXED-BED COLUMN BIOREACTOR

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RÉSUMÉ

Le microbiochar préparé à partir de bois de pin (BC-PW), de fumier de porc (BC-AS) et de coquille d'amande (BC-PW) a été évalué pour l'élimination du DCF à des concentrations environnementales pertinentes dans des conditions de fonctionnement continu. Les biochars ont été caractérisés pour la taille des particules, les métaux totaux, la composition élémentaire, la surface spécifique, la porosité, la texture de surface et les groupes fonctionnels. De plus, l'enzyme ligninolytique laccase a été immobilisée sur des microbiochars et testée pour l'élimination / biodégradation du DCF dans des conditions optimales obtenues à partir d'expériences sur colonne d'adsorption. On a trouvé que le BC-PM était un adsorbant efficace par rapport au BC-PW et BC-AS. Cette efficacité était attribuée à une plus grande surface spécifique, à la porosité et à la présence de divers groupes fonctionnels contenant des métaux. À une concentration pertinente pour l'environnement (500 µg L^{-1}), BC-PM a montré une capacité d'adsorption de 4,10 mg g^{-1} avec une masse de lit de 2 g, un débit de 2 mL min^{-1} et un pH de 6,5. Pour tous les biochars, l'absorption de DCF à travers une colonne à lit fixe dépendait de la masse de l'adsorbant (hauteur du lit) et de la concentration de DCF. La capacité d'adsorption diminue avec l'augmentation de la hauteur du lit, alors qu'elle augmente avec la concentration croissante de DCF. Les modèles de Thomas et Yoon-Nelson ont été appliqués avec succès pour prédire les courbes de percée, indiquant que ces modèles étaient utiles pour la conception et la mise à l'échelle des colonnes de biochar à lit fixe. Le biochar lié à la laccase présentait une efficacité d'élimination supérieure à celle du biochar brut et une amélioration significative dans le temps percé a été observée, qui a été attribué à la biodégradation du DCF dans la colonne.

Mots-clés: biochar; diclofénac; contaminant émergent; adsorption; colonne à lit fixe; biodégradation; laccase

ABSTRACT

Laccase bound biochar as a fixed-bed column for the removal of emerging contaminant, diclofenac(DCF) provides novel insights into valorization of waste materials, application of environmentally benign sorbents and removal of DCF with possibilities of scaling up for larger applications. Micro-biochar prepared from pine wood (BC-PW) pig manure (BC-PM) and almond shell (BC-AS) were evaluated for the removal of DCF at environmentally relevant concentrations under continuous fixed–fed column operating conditions. Biochars were characterized for particle size, total metals, elemental composition, surface area, porosity, surface texture and functional groups. igninolytic enzyme laccase was immobilized on micro-biochars and tested for removal/biodegradation of DCF under optimized conditions obtained from adsorption column experiments. BC-PM was found to be an effective adsorbent for BC-PW and BC-AS and which was attributed to the higher surface area, porosity, and presence of various functional groups containing metals. At an environmentally relevant concentration (500 $\mu g\ L^{-1}$), BC-PM exhibited an adsorption capacity of 4.10 mg g^{-1} with bed mass: 2g, flow rate: 2 mL min^{-1} and at pH: 6.5. For all biochars, uptake of DCF through a fixed-bed column was dependent on the mass of adsorbent (bed height) and DCF concentration. Adsorption capacity decreased with increasing bed height whereas increased with increasing DCF concentration. The Thomas and Yoon–Nelson models were successfully applied to predict the breakthrough curves, indicating that these models were used for designing and scaling – up fixed-bed biochar columns. Laccase bound biochar exhibited higher removal efficiency over the raw biochar and significant improvement in breakthrough time was observed which was attributed to the biodegradation of DCF in the column.

Keywords: biochar; diclofenac; emerging contaminant; adsorption; fixed-bed column; interfaces

1. Introduction

In the past decade pharmaceuticals and personal care products (PPCPs) were widely detected in environmental compartments, particularly in surface, waste and groundwater [1].. Non-steroidal anti-inflammatory drug (NSAID) diclofenac (DCF) is one of the widely detected pharmaceuticals in wastewater [2] with an estimated annual consumption of about 1443 ± 58 tons/ year [3], DCF stands among one of the top 20 generic molecules used worldwide. Diclofenac is detected in low ng L^{-1} to high µg L^{-1} concentrations in waste and surface water [4] and the ability of conventional wastewater treatment plants (WWTPs) to remove diclofenac from wastewater is rather limited [2, 4]. Diclofenac has been detected in liver, kidney, and gill of fishes and has been correlated to physiological alteration in aquatic animals even at concentrations of 1 µg L^{-1}. It is also among one of the very few drugs that can cause toxic effects to bacteria, invertebrates and algae [4, 5]. Frequent detection of DCF and its metabolites in the environment and feeble degradation/removal in WWTPs demands efficient treatment methods for DCF. Advanced treatment methods, such as membrane filtration, ozonation, and oxidation and electro-dialysis have been tested by several researchers in the past [6, 7]. However, the majority of these processes are often laden with challenges, such as high capital/ running costs and produces undesired by-/transformation products which are often toxic.

For the past several decades, adsorption has been used as one of the reliable processes for the removal of various organic and inorganic contaminants. Even with the availability of various advanced treatment methods, adsorption still remains as one of the best and efficient methods for the removal of organic contaminants owing to its inexpensive and universal nature, flexibility and ease of operation and after all its high efficiency and effectiveness [8]. Activated carbon produced from various materials was one of the most studied adsorbents for the removal of DCF [9-11]; however, the majority of these studies were carried out in batch mode. Batch mode studies cannot be considered as a standalone removal/treatment method while considering the feasibility and scaling up possibilities for higher loads of contaminated water. Handful studies so far have explored fixed bed adsorption column as a treatment method for the removal of DCF. Until date, granular activated

carbon and clay based hybrid materials fixed bed columns were reported to be effective for the removal of DCF [12, 13]. Enzymatic degradation of micropollutants has emerged as an effective tool for the degradation of several contaminants including DCF[14, 15]. Ligninolytic enzyme laccase has shown superior ability for the degradation of DCF[14]. Even though enzymes are highly effective and versatile biological catalysts their application in free form incurs challenges such as low operational stability, difficulties in recovery and reuse along with high costs. Immobilization of enzymes of solid supports has been suggested as a remedial measure to overcome these challenges[16].

Biochar is a carbon-enriched material produced from a variety of biomass by pyrolysis under oxygen-limited conditions at a relatively low temperature. Biochar typically exhibits high biochemical stability, well-developed pore structures, large surface area, and exceptional adsorption properties[17]. Moreover, due to the copious presence of various surface functional groups, biochar is known to adsorb various organic and inorganic contaminants with anionic and cationic properties [18]. Biochar as a cost-effective and value-added material for various applications is rapidly emerging as a new research domain. On this outset, studies on biochar as a fixed bed column for the removal of DCF shows the possibility of valorization of wastes and removal of DCF with possibilities of scaling up for larger volumes. Production of biochar from waste/residual materials could render fixed bed column zero-waste, cost-effective and environmentally benign. Moreover, laccase can be immobilized on biochar and thus complete removal can be expected through simultaneous adsorption of the contaminant on biochar and degradation through the immobilized laccase.

This study investigates the removal of DCF through adsorption onto micro-biochar fixed bed column. Biochar from various origins has been used as the fixed bed column. Moreover, laccase immobilized biochar has been used as the column bioreactor under optimized conditions for the effective removal of DCF. To the best of our knowledge, no similar prior study has been carried out using unmodified biochar as fixed bed column and hence entails the novelty elements of the present study. Moreover, to the best of our knowledge, no other previous study investigated on “micro-biochar fixed-bed column bioreactor” for the removal of any micropollutants.

2. Material and methods

2.1 Materials

Pinewood biochar sample was obtained from Pyrovac Inc. (Quebec, Canada) and pig manure biochar was procured from Research and Development Institute for Agri-Environment" (IRDA), Quebec, Canada. Pinewood biochar (BC-PW) was derived from pine white wood (80% v/v) spruce and fir (20% v/v) and was produced at 525±1°C under atmospheric pressure by increasing the temperature of biomass at the rate of 25 °C/min in the presence of nitrogen for 20 minutes. Pig manure biochar (BC-PM) was derived from the solid fraction of pig slurry and prepared at 400±1 °C for 2 h. Biochar obtained from reactor outlet was non-uniform and bigger in size (about 2- 3 mm). The biochar was thus ground and sieved to get a uniform size distribution. Particles were ground and sieved through ASTM no: 200 sieves (corresponds to 75 µm mesh size) to obtain a uniform size distribution of micron level. Through grinding, particle size reduced and therefore surface area was also increased [19] which in turn resulted in increased active sites for adsorption. Moreover, according to the International Union of Pure and Applied Chemistry (IUPAC) guideline- "Terminology for related polymers and applications (IUPAC Recommendations 2012, [19] microparticles are considered to be particles with diameters between 1-100 µm. Further, application of nanoparticles can enhance the efficiency of the system in fixed bed column mode however often difficult to operate due to difficulties, such as leaching of particles during operation and heavy resistance and pressure drop inside the column. Moreover, "toxic concerns" regarding nanoparticles still remains as an unattended problem [20]. Thus, micro-sized particles were selected as the suitable material for molding fixed bed columns for this study. For preparing microparticles, samples were ground by 'Retsch RS 200' vibratory disc mill at 750 rpm for 2 minutes and sieved through ASTM 200 numbered sieve (corresponds to less than 75µm particle size) for 10 minutes.

Apple industry residue, apple pomace was used as the substrate for laccase production and *Trametes versicolor* (ATCC 20869) was used as fungi for the production of laccase. Details

regarding laccase production have been published before. [21] After extraction, laccase has been concentrated using Sartorius stedim − SartoJet ultrafiltration system (Göttingen, Germany). About 1.1 U mL^{-1} of laccase activity was obtained after concentration.

Prefabricated Bio-Rad chromatography columns (1.0 × 20 cm; Econo-column) were used as column beds for filling biochar. Diclofenac sodium salt (98%; CAS 15307-79-6) was purchased from Fisher Scientific (Ottawa, ON, Canada) and internal standard (IS), diclofenac-d_4 was obtained from C/D/N Isotopes Inc. (Montreal, QC, Canada). Adsorption experiments were carried out in Milli-Q water and prepared using Milli-Q/Milli-RO Millipore system (Milford, MA, USA). Wastewater collected from tertiary treatment stage of Quebec Urban Community wastewater treatment plant (spiked with 500 µg L^{-1} of DCF) was used for testing the biodegradation column.

2.2 Methods

2.2.1 Characterization of biochar, enzyme assay, and analysis of diclofenac

Particle size distribution of raw and biochar microparticles were measured using Horiba particle size analyzer (LA-950 Laser Particle Size Analyzer, Horiba, Japan. Scanning electron micrographs were captured using EVO® 50 smart scanning electron microscope (SEM) (Zeiss, Germany). Specific surface area and porosity of the microparticles were obtained from Brunauer, Emmett and Teller (BET) N_2 adsorption isotherms at 77 K (Autosorb-1, Quantachrome, USA). Surface functional groups on the microparticles were identified by Nicole iS50 FT-IR Spectrometer (Thermo Scientific, USA). Elemental analysis of micro-biochar was carried out using TruSpec® Micro- CHN analyzer (Michigan, USA). Metals in biochar were quantified by ICP-AES (Varian, California; USA). Other characterization details regarding the biochars used were given in previous publications [19, 22].

Laccase activity was measured at 420 nm using 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Detailed procedure has been given in the previous publication[14]. Analysis of DCF was performed by LDTD-MS/MS technique as described by Lonappan et. al [2].

2.2.2 Fixed bed column adsorption studies

Column experiments were conducted using a glass column (1 cm inner diameter, 20 cm long) packed with biochar (2 g and 5 g) and sealed with glass wool below and above and then the column was packed with glass beads (3mm diameter; Fisher Scientific, Ottawa, ON, Canada). This measurement confirmed compactness of adsorbent and removed the possible pressure drop and dead volumes inside the column. Diclofenac sodium solution (prepared and stored in an amber reservoir of 4 L capacity) was pumped from the top of the column using a peristaltic pump. Various DCF concentrations were tested (from 500 ug L^{-1} to 5 mg L^{-1}) and the concentration chosen (500 ug L^{-1}) was close to the concentration of DCF which was observed in some wastewaters. A constant flow rate of 2mL min^{-1} was used throughout the study. pH (6.5) and temperature (25± 1 °C) was kept constant throughout the experiment by preparing solutions in milli-Q water and by conducting experiments at room temperature. Effluents were collected at various time intervals and centrifuged (if necessary) at 11600 *x g* for 10 minutes in a MiniSpin® plus centrifuge for removing any traces of biochar. Samples were analyzed for remaining DCF in the liquid.

Performance indicators of the fixed-bed column can be calculated from the following equations [23, 24]. The loading behavior of biochar onto the fixed-bed column was expressed in terms of the normalized concentration C_0/C_t (where C_0 and C_t are the inlet and outlet (at time t) DCF concentrations, respectively) for a given mass of biochar (bed height) and corresponds to breakthrough curve. Effluent volume can be calculated using the Equation 1.

$$V_{eff} = Qt_{total} \quad (1)$$

Where, t_{total} and Q are the time of exhaustion (hour) and volumetric flow rate (mL min^{-1}), respectively. For a fixed DCF concentration and constant flow rate, the total area above the breakthrough curve can be calculated by integrating the total adsorbed concentration, C_{ad} (mg L^{-1}) Vs t (hours) plot and can be used to find total adsorbed DCF capacity, q_{total} (mg)as given in Equation 2:

$$q_{total} = \frac{Q}{1000} \int_{t=0}^{t=t_{total}} C_{ad}\, dt \qquad (2)$$

The equilibrium uptake q_{eq} (μg g^{-1}) of the column was calculated from Equation 3:

$$q_{eq} = \frac{q_{total}}{x} \qquad (3)$$

Where x (g) is the total dry weight of biochar in the fixed-bed column. The total amount of DCF adsorbed into the column, W_{total} (mg) was calculated from Equation 4:

$$W_{total} = \frac{C_t \;\; Qt_{total}}{1000} \qquad (4)$$

The removal percent of DCF at saturation can be obtained from Equation 5:

$$R(\%) = \frac{q_{total}}{W_{total}} * 100 \qquad (5)$$

Finally, breakthrough adsorption capacity can be calculated from the following Equation 6:

$$q_b = \left\{\frac{C_i - C_b}{x}\right\} b_v \qquad (6)$$

Where, Ci and C_b are the initial and breakthrough concentrations (mg L^{-1}); b_v is the breakthrough volume of DCF solution in liters and x is the mass of the adsorbent used (g).

2.2.3 Microbiochar fixed-bed column bioreactor

For immobilization of laccase on microbiochars, about 100 mL of crude laccase (1.1 U mL^{-1} activity) was mixed with 10 g of microbiochar for 5 h followed by the addition of glutaraldehyde (5% *v/v*). This mixture was further shaken for another 7 hours h (at room temperature, 25 °C). Later, the laccase-biochar mixture was centrifuged and biochar was removed. The supernatant was analyzed for enzyme activity and theoretical binding of laccase on biochar was calculated. Laccase immobilized biochar was dried at room temperature and used for column packing. About 5 g biochar was used for the column packing. Wastewater (effluent from wastewater treatment plant) spiked

with 500 µg L^{-1} of DCF was used for testing the biodegradation column. Experiments were carried out under optimized conditions which were obtained from the adsorption studies. Further experiments were carried out as described in section 2.2.2.

3. Results and discussion

3.1 Characterization of biochars and immobilization of laccase on microbiochars

Particle size distribution of biochars as obtained and micro-biochars are given as Fig. 1. For raw BC-PW, mean particle size was recorded to be 942.5 ± 438.8 µm, whereas, for raw BC-PM, mean particle size was ≈482.1µm. Through grinding and sieving, the particle size was reduced to ≈36.3 µm and ≈30.6 µm, respectively for BC-PW and BC-PM. Thus, biochar used to fill adsorption columns was “micro-biochar” according to IUPAC guidelines.

Obtained specific surface area of micro biochars was 13.3 m^2 g^{-1} and 43.5 m^2 g^{-1}, respectively for BC-PW and BC-PM. The total pore volume of micro BC-PW was obtained as 0.0208 mL g^{-1} for pores smaller than 5650.9 Å (diameter), (at P/Po = 0.99660) and pore width (mode value) was obtained as 27.69 Å. Previous studies also reported similar results for biochar prepared from pine wood through fast pyrolysis [25]. Moreover, BET experiments resulted in a type-1 isotherm which corresponds to microporous solids [25]. Unfortunately, porosity analysis failed for micro BC-PM samples and a further increase in degassing temperature was not useful since samples started thermal decomposition. Both CO_2 and N_2 were not able to replace already occupied sorption sites and thus pointed towards the possibility of irreversible chemisorption. This was further confirmed by the TGA profile which is given in figure 2. It was clear that thermostability of BC-PM was lower in comparison with BC-PW. However, further analysis by scanning electron microscope revealed the porous nature of biochars and thus confirmed that micro BC-PM too has a porous nature. Scanning electron micrographs of BC-PW and BC-PM are given as Fig. 3. SEM photographs of biochar samples showed rough and uneven surfaces with the presence of several pores and can effectively sorb DCF.

The FTIR spectra of biochars are given in Fig. 4. For pinewood biochar (BC-PW), after pyrolysis, some hydroxyl moiety remains in sample [26] and the sharp peak near 3400 cm^{-1} suggested the presence a free hydroxyl group. Due to the plant source origin of BC-PW presence of lignin/cellulose-derived transformation products was observed [17] (multiple peaks starting around 1600 cm^{-1} to 700 cm^{-1}). Another peak at 1584 cm^{-1} indicated aromatic ring modes which are characteristic of lignocellulose materials, such as Pinewood (softwood) [27]. A strong peak at 1197 cm^{-1} was attributed to phenolic C-O bond. The peak at 750 cm^{-1} was due to aromatic C-H bend. The FTIR spectra of pig manure biochar (BC-PM) suggested complex functional groups consisting of a mixture of minerals and organic matter. For pig manure biochar, a narrow peak at 3400 cm^{-1} indicated the presence of secondary amines. The peak at 2533 cm^{-1} attributed towards the presence of mercaptans (S-H stretch). A broad and strong peak of around 1422 cm^{-1} indicated the presence of aromatics (C–C stretch). Peak around 1114 cm^{-1} was attributed to C-O stretch and signifies the presence of carboxylic acids. Peaks around 874 cm^{-1} and 563 cm^{-1} can be assigned to PO_4^{3-} group. The peak around 712 cm^{-1} could be due to the presence of calcium and peak around 617 cm^{-1} could be due to the presence of disulfides (S-S) stretching. In addition, peak around 563 cm^{-1} was attributed towards the presence of alkyl halides.

Further, an attempt to quantitatively analyze results obtained from FTIR was made through analysis of samples for elemental composition and metals. Functional groups and presence of certain metals as well as oxygen can affect adsorption [28]. The chemical composition of biochar microparticles is given as Table 1. In comparison with BC-PW, total carbon was very low in BC-PM and was about 58 %. In BC-PW, total carbon constituted about 84 % and the presence of inorganic materials in very small quantities made BC-PW as an organically rich adsorbent. Total hydrogen and nitrogen constituted about 4.5 % in composition. In addition, about 1-2 g/kg of Ca, Fe and K was also present in BC-PW. In comparison with BC-PW, biochar derived from pig manure (BC-PM) showed the abundant presence of metals through ICP analysis and which was also confirmed by FTIR analysis. Usually, biochar from pig manure feedstock contains metals in high concentrations due to its swine

manure origin [29-31].Very high concentrations of Ca(about 45.5 g/kg) was observed in BC-PM followed by P (about 22.6 g/kg) , K (about 21 g/ kg) , Fe (about 10g/kg), Mg (about 10.1 g/kg), S(about 8.4 g/kg) and Na (8 g/kg). Thus, pig manure biochar was rich with inorganic compounds as already reported by previous studies [30, 31]

BC-PW resulted with 0.7 U g^{-1} of laccase whereas, for BC-PM it was 0.9 U g^{-1}. Laccase was covalently immobilized onto the microbiochars with the help of crosslinking agent glutaraldehyde. Further, laccase immobilized microbiochars were used for the packing of the column.

3.2 Adsorption of diclofenac onto biochars

BC-PM exhibited enhanced adsorption potential over BC-PW. For a DCF concentration ≤500 ug L^{-1}, the flow rate of 2 mL min^{-1} and biochar mass of 2 g BC-PM about 89 % DCF was removed in the first 30 minutes, whereas for BC-PW, the removal was about 60%. Previous studies have also reported the enhanced capacity of pig manure biochar for the removal of various contaminants [19, 29]. Under fixed-bed continuous flow conditions, initially, biochar at the top of the column gets saturated and maximum removal takes place during the beginning which was reflected in these results. The adsorption zone moves downward with time and reaches the exit of the bed and at this time, the concentration of DCF in the effluent becomes equal to the influent. A plot of effluent and influent concentration ratios (C_t/C_0) versus elapse time (t) will result in 'breakthrough curve'. The enhanced adsorption capacity of BC-PM over BC-PW can be correlated with the higher surface area of BC-PM. Moreover, apart from higher surface area, the presence of metals, such as Ca and Mg can enhance DCF adsorption[29] by forming precipitates. In this study, elemental analysis and metal analysis (table 1) showed that Mg (g/kg) (PM- 10.1, PW- 0.14) and Ca (PM- 45.4 and PW- 1.7) concentrations are much higher in PM than PW and thus formed precipitates which in turn resulted in enhanced adsorption. Another possible reason can be the porous structure of biochars. With the micro-sized pores, sieving effects can be observed for DCF due to its relatively larger structure [32].

Thus, with increased ratios of larger sized pores, DCF can get better contact with biochar surface in comparison with other carbonaceous adsorbents [33], resulting in enhanced adsorption.

3.3 Fixed bed adsorption columns under various experimental conditions

3.3.1 Effect of initial DCF concentration

Keeping flow rate and mass of biochar constant at 2 mL min^{-1} and 2 g, respectively, the effect of two initial DCF concentrations (0.5 mg L^{-1} and 5 mg L^{-1}) were studied and breakthrough curves are presented as fig.5. The breakthrough time decreased with increasing influent DCF concentration. At lower and environmentally relevant initial DCF concentration (0.5 mg L^{-1})[5] the breakthrough curves were spread in a wide range and the 'breakthrough rate' was slower. With increased initial DCF concentration, the slopes become steep in breakthrough curves owing to the faster mass transfer flux from the bulk solution to the particle surface as a result of the increased diffusion coefficient. This phenomenon is much more visible for BC-PM than BC-PW. However, for both biochars, breakthrough curves were not very steep which indicated that the exhaustion of the bed was relatively slow. For BC-PW, a breakthrough was obtained at the 18^{th} hour for 0.5 mg L^{-1} DCF and at the 5^{th} hour for 5 mg L^{-1} DCF. For BC-PM, a breakthrough was obtained at the 24^{th} hour for 0.5 mg L^{-1} DCF and at the 8^{th} hour for 5 mg L^{-1} DCF. Thus, for both the biochars, a change in concentration gradient affected the saturation rate and the time taken to achieve a breakthrough. Generally, high initial concentrations of the adsorbates can enhance column performance by increasing the driving force and hence decreasing the length of the adsorption zone [24, 34] and a similar phenomenon was observed in this study as well.

3.3.2 Effect of mass of biochar (bed height)

The effect of the mass of biochar (2 g or 5 g) on the breakthrough curve at a constant initial DCF concentration (0.5 mg L^{-1}) and flow rate (2 mL min^{-1}) is shown in Fig. 6. For BC-PM, 2g biochar corresponds to 3.5cm column height while 5 g corresponds to 8 cm. Whereas, for BC-PW 2g biochar

resulted in a column height of 5cm; while 5g resulted in 12.5 cm. The increase in the total amount of adsorbents resulted in an increase in total available adsorption sites[24]. Thus, for biochars, breakthrough, and saturation times were increased. For BC-PW, a breakthrough was obtained at the 18th hour for 2 g of biochar and at 30th hour for 5 g of biochar. For BC-PM, breakthrough obtained at the 24th hour for 2 g of biochar and at the 36th hour for 5 g of biochar. An increase in adsorption capacity and saturation time was observed for both biochars with an increase in bed height (mass of biochar). This can be attributed to the longer residence time of DCF inside the column and hence better interactions (further diffusion) between biochar and DCF [24, 34].

3.4 Dynamic modeling of breakthrough curves

For further scaling up, and design and continued successful operation of the fixed-bed column, the exact determination of breakthrough curve is necessary. Thus, breakthrough curves for the adsorption of DCF onto biochars were analyzed using the Thomas, Yoon–Nelson and Adams–Bohart, models to determine the dynamic behaviors in the column. The sorption performance of DCF through the column was analyzed by Thomas, Yoon-Nelson, and Adams –Bohart models at starting ratio of C_t/C_0 >0.1 (10% breakthrough) until C_t/C_0 >0.90(90% breakthrough) [35].

3.4.1 Thomas model

Thomas model is useful in determining the maximum solid phase concentration of the solute on the adsorbent and calculating the adsorption rate constant for the adsorption column and the Thomas model can be written as Equation 7[36]

$$\frac{C_t}{C_0} = \frac{1}{1+exp\{(k_{th}\,(q_0 m - C_0 V_{eff})/Q\}} \qquad (7)$$

Where k_{th} is the Thomas rate constant (mL/min.g), q_0 is the equilibrium adsorbate uptake (mg/g), Q is the volumetric flow rate(mL/min), V_{eff} is the volume of effluent (mL), and m is the mass of adsorbent in the column (g).

The Thomas rate constant and adsorption capacity of biochars were plotted and calculated according to Eq. (7), and the results are presented in Table 2. It was observed that Thomas model's correlation coefficients were high for both BC-PW and BC-PM (R^2 > 0.95; except for 5 g adsorbent mass for BC-PM) and which indicates that the model is able to describe the dynamic behaviors in the fixed-bed column. With an increase of initial DCF concentration, the value of K_{Th} decreased for both biochars and this is because of variable concentration conditions, and that the driving force for the adsorption is the concentration difference between DCF and biochar [35, 37].Moreover, for both BC-PM and BC-PW, a significant ($p < 0.05$) increase in q_0 (adsorption potential or bed capacity) was observed with an increase in DCF concentration due to the concentration difference between DCF and biochar. From q_0 values, it is clear that BC-PM is better adsorbent than BC-PW. With an increased mass of biochar (bed height), the value of K_{Th} increased for both biochars, whereas bed capacity q_0 decreased for both biochars. The perfect fitness of the experimental data with Thomas model showed that diffusion (both external and internal) cannot be the rate-limiting parameters[35].

3.4.2 Yoon-Nelson model

The Yoon–Nelson model requires no detailed data about the characteristics of the adsorbent physical properties in the fixed-bed column [38]. Yoon–Nelson model is expressed as Equation 8:

$$\frac{C_t}{C_0} = \frac{1}{1+exp\{(k_{YN}\,(\tau - t)\}} \qquad (8)$$

Where, K_{YN} is the Yoon–Nelson rate constant (h^{-1}), and τ is the time required for the column to reach 50% adsorbate breakthrough based on the Yoon–Nelson model. k_{YN} is an important parameter in fixed –bed column dynamics and it depends on the diffusion characteristics of the mass transfer zone.

The Yoon–Nelson rate constant and time required for 50% adsorbate breakthrough for biochars is calculated according to Eq. (8), and the results are presented in Table 2. The Yoon–Nelson's model correlation coefficients were higher for both BC-PW and BC-PM (R^2 > 0.95; except for 5 g adsorbent

mass for BC-PM) and which indicates that the model can explain 95% of the variability observed regarding the diffusion in the fixed bed column. The time required to reach 50% adsorbate breakthroughs (τ) were higher for BC-PM than BC-PW and hence BC-PM was better adsorbent under fixed-bed column conditions for removing DCF. Moreover, an increase in k_{YN} values with increase in DCF concentration and bed height proved that the column was saturated with DCF at a higher rate under these conditions. Moreover, for both biochars, the column saturation reached much faster with increased DCF concentration. This could be due to the increased availability and as a result, elevated interactions of DCF with biochar. Previous studies also observed the increased interaction of adsorbate resulting in a better adsorption [9, 34]. For BC-PW, an increased column height increased the τ, whereas, for BC-PM, the τ value was reduced by about 5 %.

3.4.3 Adams- Bohart model

Adams- Bohart developed this model based on surface reaction theory and presumed that equilibrium is not instantaneous. This model describes the initial part of the breakthrough curve. According to this model, the rate of adsorption is the proportional to residual capacity of the adsorbate bed and concentration of the sorbate. Linearized form of Adams- Bohart model can be written as Equation 9:

$$ln\left(\frac{C_t}{C_0}\right) = K_{AB}\, C_0 t - K_{AB}\, N_0 \left(\frac{z}{U_0}\right) \qquad (9)$$

Where C_0 and C_t are the inlet and outlet adsorbate concentrations respectively, z (cm, g) is the bed height (mass) , U_0 (cm min^{-1}) is the superficial velocity. N_0 (mg L^{-1}) is the saturation concentration and K_{AB} (L $mg^{-1} min^{-1}$) is the mass transfer coefficient.

The saturation concentration and mass transfer coefficient for biochars were calculated from Eq. (9), and the results are presented in Table 2. In comparison with the Thomas and Yoon-Nelson models, R^2 was lower which suggested that this model cannot be applied for explaining adsorption dynamics of DCF onto BC-PW and BC-PM.

It is clear that both Thomas and Yoon-Nelson model explained the adsorption behavior of both biochars for DCF. Thomas model is based on Langmuir kinetics of adsorption-desorption and no axial dispersion is derived with the assumption that the rate driving force obeys second-order reversible reaction kinetics [36]. Thus, while designing scaling up a column for removing DCF using pine wood and pig manure biochars second order reversible kinetics can be observed. The column design parameters as obtained in table 1 could be used for the design of adsorption column for a practical purpose which can further used for larger volumes.

3.5 Microbiochar fixed-bed column bioreactor

Along with adsorption continuous biodegradation was observed with microbiochar fixed-bed column bioreactor. An initial concentration of 0.5 mg L^{-1} of DCF was used and a constant 2 mL min^{-1} flowrate and 2 g of laccase immobilized microbiochar was used was used. For BC-PM, 2 g laccase immobilized microbiochar corresponded to corresponds to 4.1 cm and for BC-PW 2 g laccase immobilized biochar corresponded to 6 cm.

Similar to microbiochar fixed bed adsorption column removal efficiency gradually decreased over time. Figure 7, presents the results regarding the microbiochar fixed-bed column bioreactor. Near 70% removal was observed with BC-PW column bioreactor whereas it was near 60 % with the adsorption column. Similarly, 88% removal was observed with laccase bound BC-PM column even though a similar removal was observed with the adsorption column as well. Laccase-mediated degradation is a slow process and it reported that V_{max} of DCF degradation though laccase mediated process is reported to 80.6 μg L^{-1} h^{-1} under batch mode conditions [14]. Thus being a slow process, degradation of DCF can occur eventually after adsorption. As a result, huge improvement in removal efficiency was not observed however, immobilized enzymes can degrade DCF in an eventual process. Thus, with biodegradation column, complete elimination DCF can be expected instead of mass transfer which is occurring in the adsorption column. Similar results (near 100% DCF removal) was observed with laccase bound activated carbon column bioreactor in a previous study[39].

4. Conclusions

Biochar prepared from pig manure can be used as an effective adsorbent over biochar prepared from pinewood for the effective removal of DCF. For both biochars, uptake of DCF through a fixed-bed column was dependent on the mass of adsorbent (bed height) and DCF concentration. At an environmentally relevant concentration (500 $\mu g\ L^{-1}$), BC-PM exhibited an adsorption capacity of 4.10 $mg\ g^{-1}$ with bed mass: 2g, flow rate: 2 $mL\ min^{-1}$ and at pH: 6.5. For both biochars, the adsorption capacity was decreased with increasing bed height whereas it increased with increasing DCF concentration. For pig manure biochar, the breakthrough was obtained at the 24^{th} hour for 2 g of biochar and at the 36^{th} hour for 5 g of biochar; whereas, for pinewood biochar, the breakthrough was obtained at the 18^{th} hour for 2 g of biochar and at 30^{th} hour for 5 g of biochar. Thus, column sorption performed better at higher influent DCF concentration and lower bed height. The surface area, porosity, and presence of various functional groups containing metals enhanced the adsorption capacity for BC-PM over BC-PW. For both biochars, second-order reversible reaction was observed for the adsorption of DCF. The modeling parameters can be successfully used for further scaling the process. Application of biochar has tremendous potential for the removal of DCF from wastewater in comparison with other commonly used adsorbents. Laccase bound biochar exhibited slightly improved removal efficiency over the raw biochar and improvement in breakthrough time was also observed which was attributed to the biodegradation of DCF in the column

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Table 1: Chemical composition of pinewood (BC-PW) and pig manure (BC-PM) biochars

Element	BC-PW	BC-PM
% C	83.6±2	57.8±1
% H	3.5±0.7	1.5±0.1
% N	< 1	3.0±0.2
Metals	**BC- PW (mg/kg)**	**BC- PM (mg/kg)**
Al	77.9±3.2	385.2±13.2
As	0.5±0.05	0.9±0.1
B	15.1±1.2	121.9±2.2
Ba	32.5±1.2	37.6±3.2
Ca	1753.3±14.5	45481.8±26.8
Cd	0.3±0.15	0.9±0.2
Co	9.3±1.2	5.6±0.5
Cr	170.6±1.6	8.1±1.6
Cu	5.2±0.7	434.8±15.7

Fe	1488.9±12.8	10638.2±16.7
K	1709.6±78.4	21115.2±165.2
Mg	143.4±10.2	10111.5±78.6
Mn	124.6±1.9	563.1±23.8
Mo	0.60±0.1	2.79±0.3
Na	100.2±12.8	8005.5±12.7
Ni	54.6±0.5	5.1±0.4
P	121.9±1.7	22695.1±3.5
Pb	4.7±1.5	14.10±1.4
S	109.9±6.7	8412.0±88.3
Sb	4.3±0.2	3.2±0.3
Se	0.4±0.1	5.4±1.2
Sn	216.6±6.5	92.9±4.5
Zn	24.4±0.2	736.3±6.5

Table 2: Model parameters of fixed-bed column adsorption of DCF onto biochars; (BC-PW) and pig manure (BC-PM) biochars

Biochar	C_0 (mg L^{-1})	Mass (g)	Thomas Model			Yoon-Nelson model			Adams - Bohart model		
			k_{TH} (L/mg/h)	q_0 (mg/g)	R^2	k_{YN} (h^{-1})	τ (h)	R^2	k_{AB} (L/mg/h)	N_0 (mg/L)	R^2
BC-PW	0.5	2	0.568	1.106	0.979	0.291	2.340	0.979	0.078	8.858	0.727
	5	2	0.126	5.174	0.988	0.634	1.028	0.988	0.023	32.130	0.803
	0.5	5	0.756	0.657	0.983	0.268	6.507	0.983	0.140	1.80	0.625
BC-PM	0.5	2	0.480	4.108	0.980	0.240	8.216	0.980	0.172	9.831	0.867
	5	2	0.150	16.147	0.974	0.757	3.199	0.974	0.604	3.254	0.789
	0.5	5	0.640	0.815	0.820	0.325	8.027	0.820	0.258	1.720	0.534

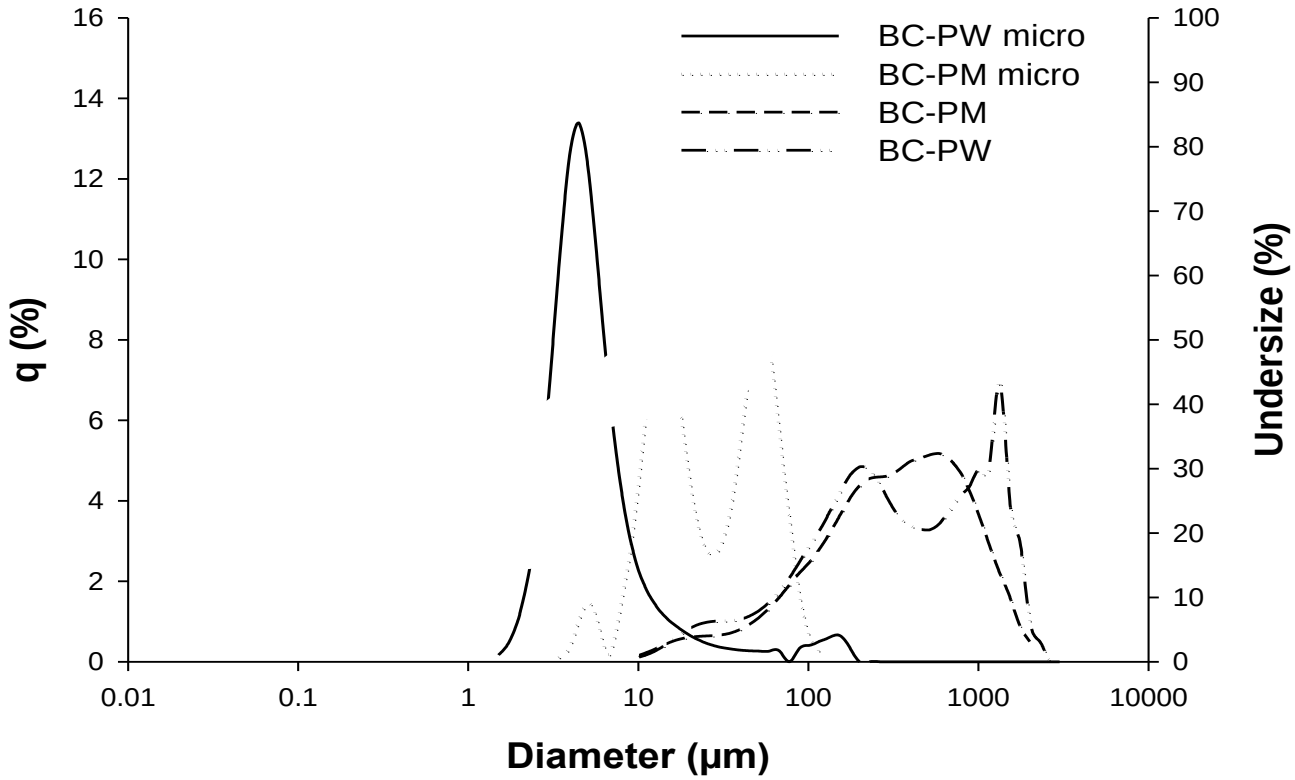


Figure 1: Particle size distribution of biochar

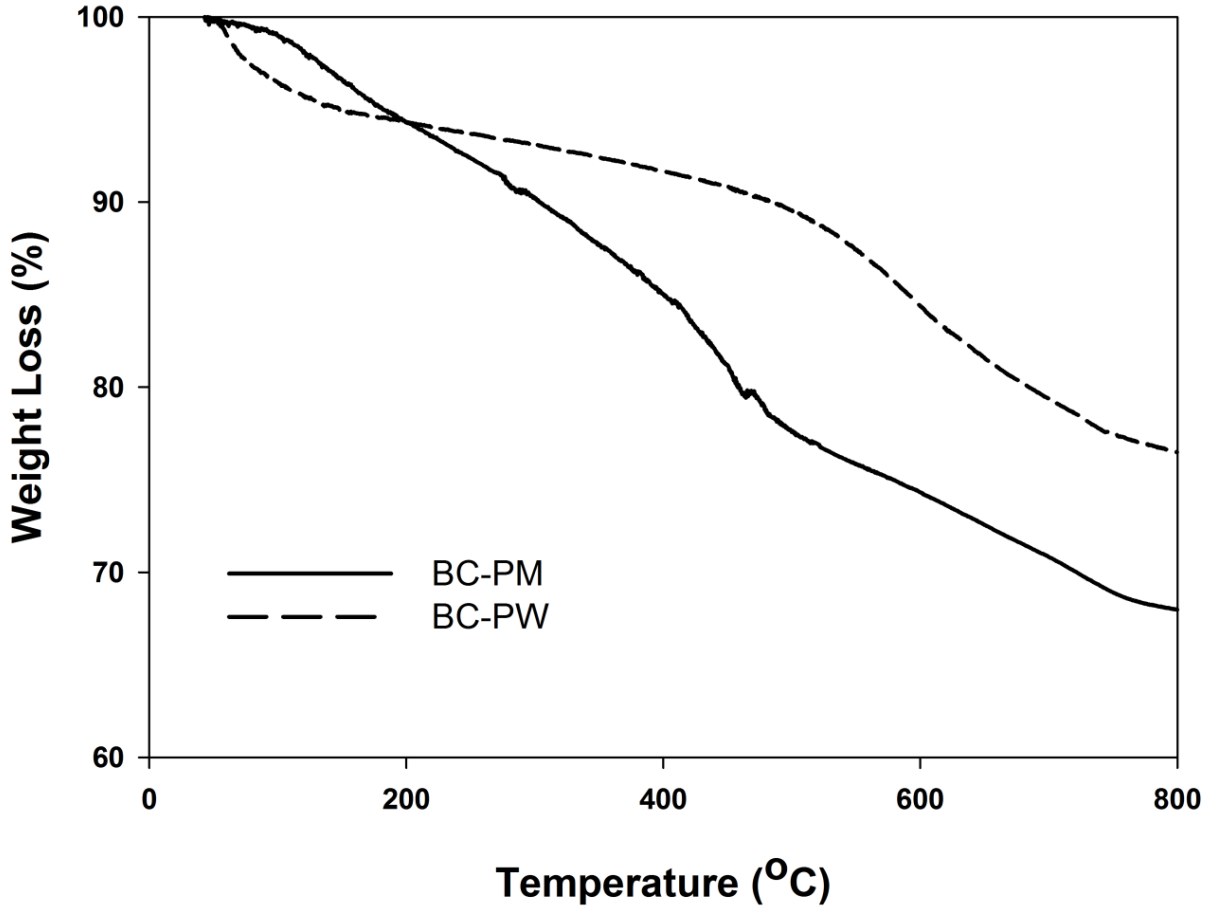


Figure 2: Thermogravimetric profiles of biochar: Pinewood biochar (BC-PW), Pig Manure biochar (BC-PM)

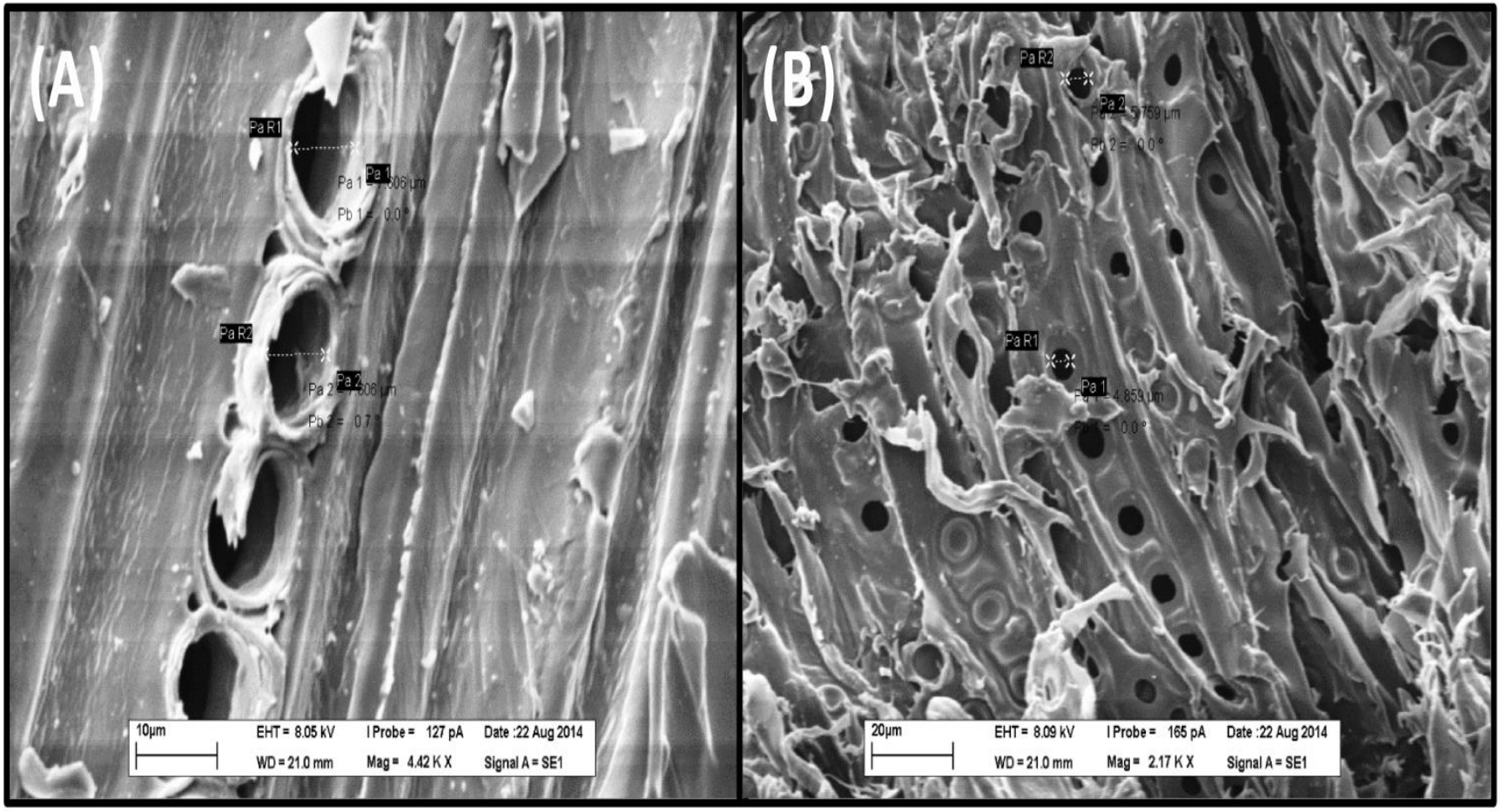


Figure 3: Scanning electron micrographs of biochar: (A) Pinewood biochar (BC-PW), (B) Pig Manure biochar (BC-PM)

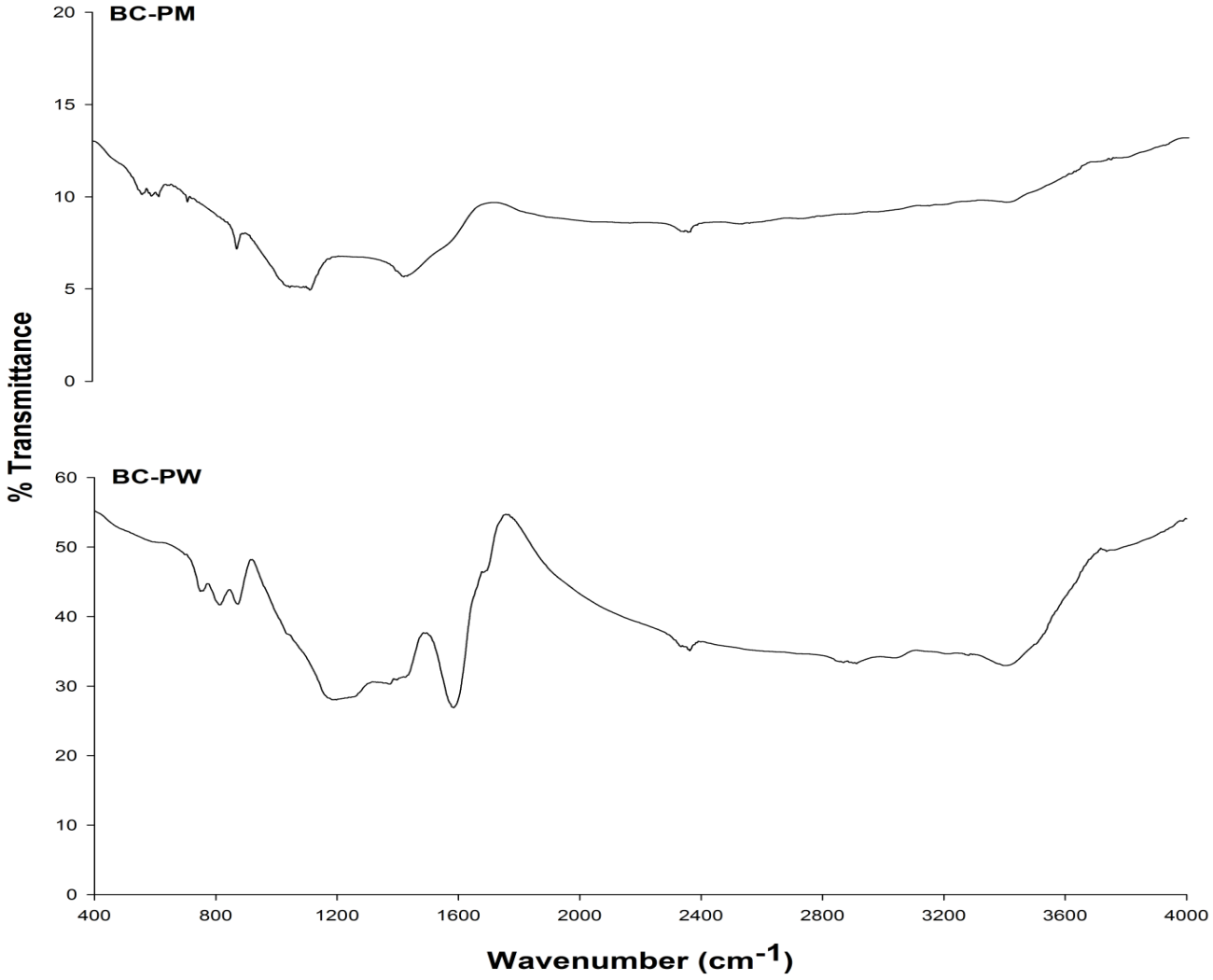


Figure 4: Fourier transform infra-red spectra for biochar- pine wood biochar (BC-PW) and pig manure biochar (BC-PM)

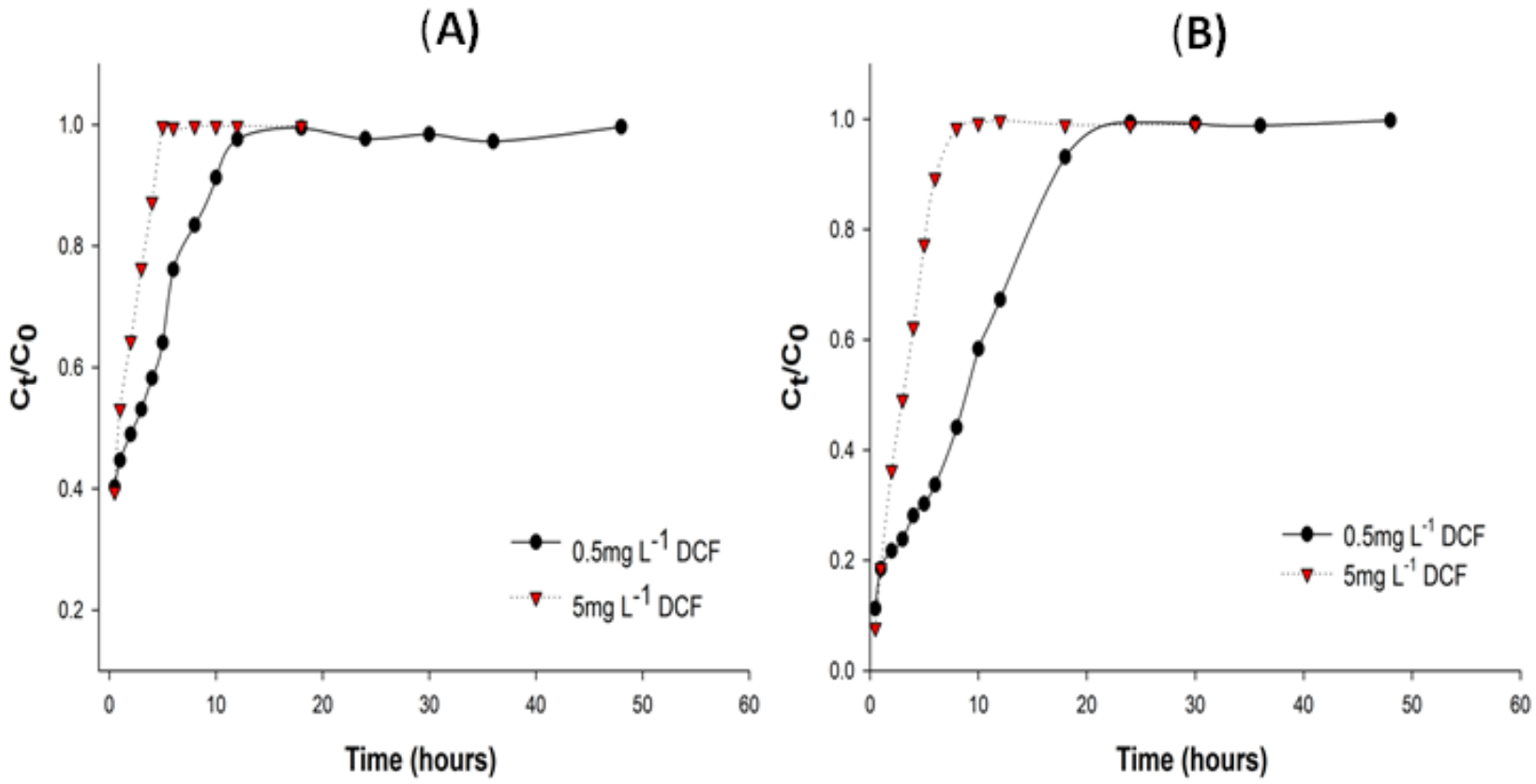


Figure 5: Breakthrough curves with different initial diclofenac concentrations: (A) pinewood biochar (BC-PW) and (B) pig manure biochar (BC-PM)

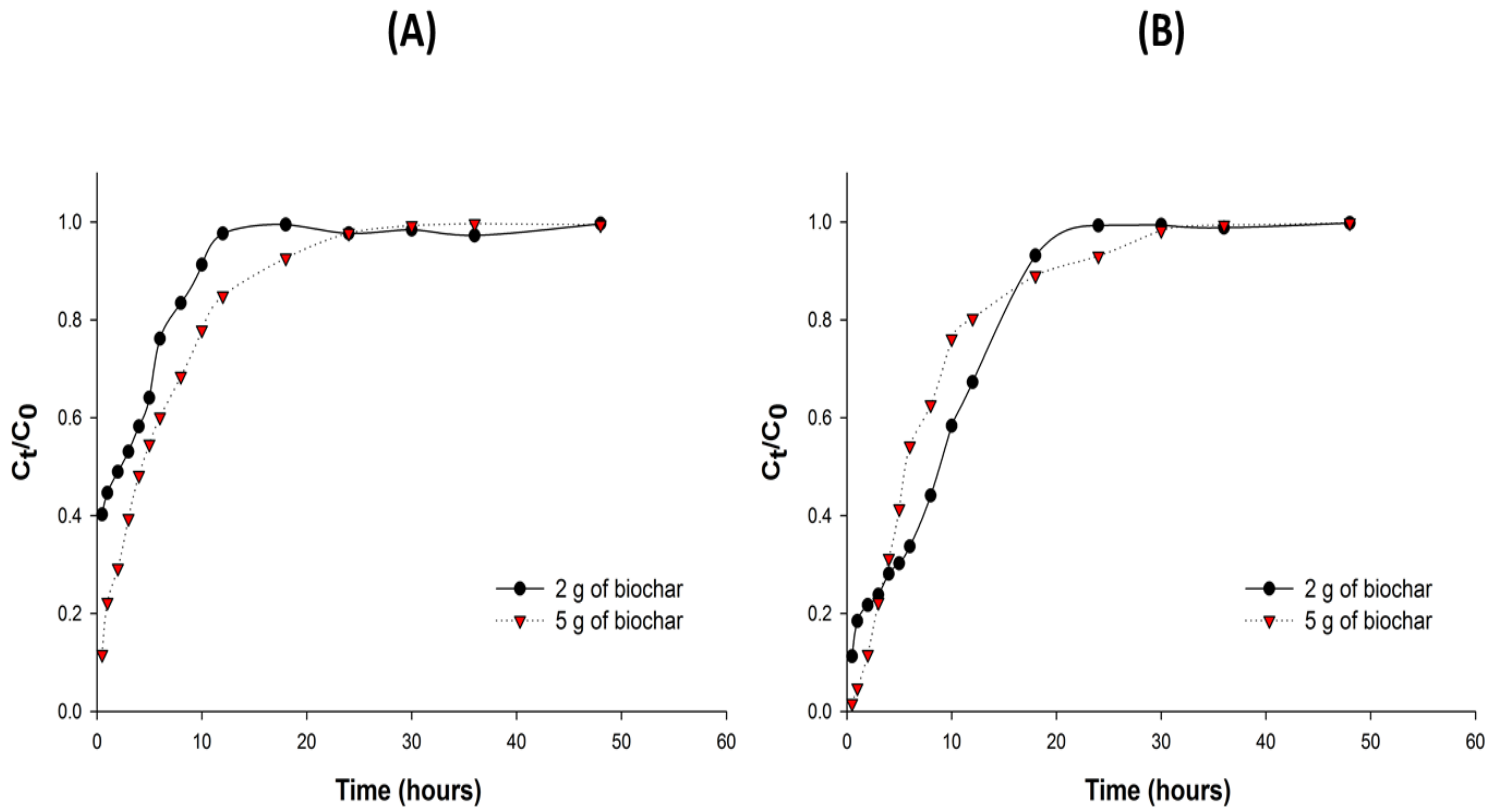


Figure 6: Breakthrough curves with different bed heights: (A) pine wood biochar (BC-PW) and (B) pig manure biochar (BC-PM)

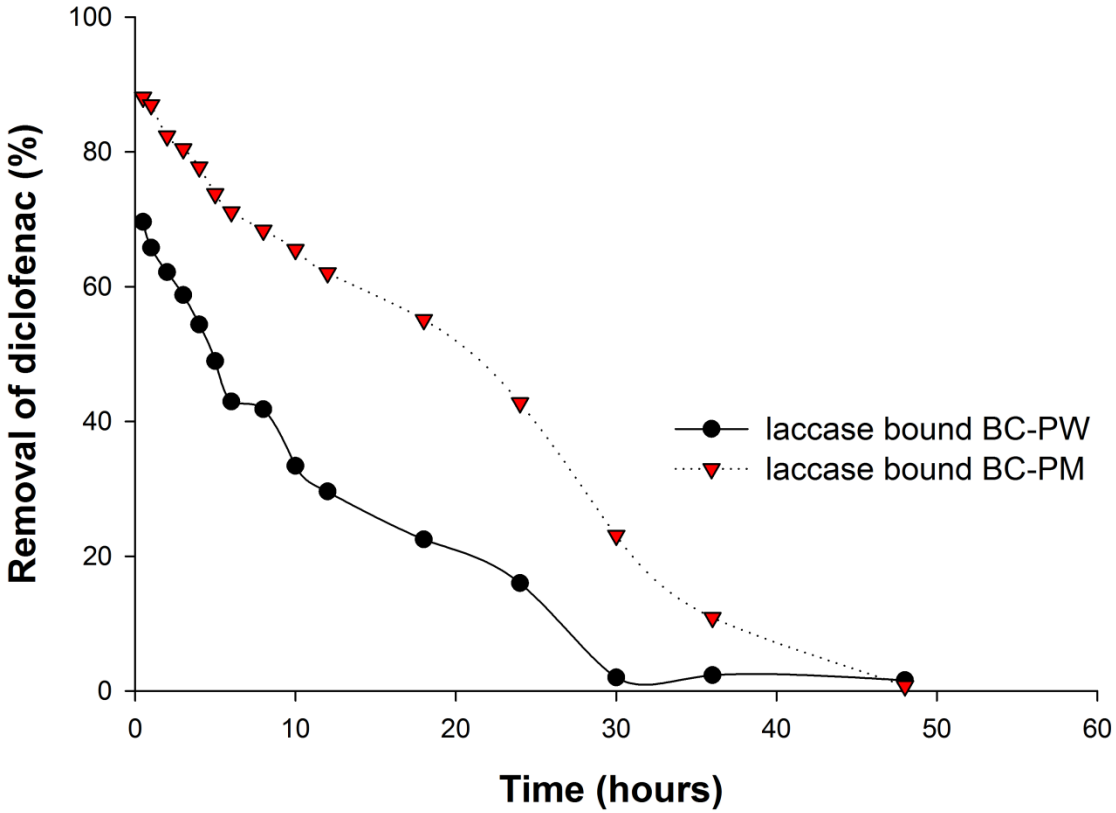


Figure 7: Degradation of diclofenac in microbiochar fixed bed column bioreactor

CHAPTER 6

CONCLUSIONS ET RECOMMANDATIONS

CONCLUSIONS

Les conclusions suivantes ont été tirées des travaux de recherche:

1. Procédé de matrice à tendance rapide pour la quantification du DCF : la nouvelle méthode développée LDTD-APCI-MS / MS a réduit le temps d'analyse par des échantillons à 12 secondes au lieu de 12 minutes pour LC-ESI-MS / MS. Les limites de détection de la méthode LDTD-APCI-MS / MS ont été entre 270 ng L^{-1} (LOD) et 1000 ng L^{-1}. La méthode d'extraction accélérée par solvant (EAS) s'est révélée plus efficace que l'extraction assistée par ultrasons (EAU) pour l'extraction du DCF des boues d'épuration avec une récupération de 95,6 ± 7 %. En effet, l'extraction du DCF à partir de boues des eaux usées avec 95,6 ± 7% de récupération. La station d'épuration des eaux usées de la ville de Québec contenait 64,89 ± 6.7µg L^{-1} de DCF et l'effluent contenait 15,95 ± 3.7µg L^{-1}. L'accumulation de DCF dans les boues primaires (1,10 ± 0,15 µg g^{-1}), ainsi que des boues secondaires (0,90 ± 0,15 µg g^{-1}) a été observée dans les stations d'épuration. Les effluents destinés pour être rejeter dans le fleuve Saint-Laurent (dans ce cas, des stations d'épuration des eaux usées du Québec) contenait des concentrations élevées de DCF, ce qui exige le développement de méthodes de traitement efficace pour l'élimination complète du DCF.

2. Les microparticules de biochar ont montré une capacité importante d'adsorption. La sélection de matières premières agricoles et la méthode de production ont eu des effets significatifs sur les propriétés du biochar, ainsi que sur sa capacité optimale d'adsorption. À des faibles concentrations de bleu de méthylène, le microbiochar a montré une capacité d'adsorption comparable à celle du charbon actif. Cela peut encore être extrapolé pour l'élimination des contaminants émergents, car ils sont généralement des micropolluants.

3. Le biochar du fumier de porc a montré une capacité d'adsorption pour la DCF supérieure à celle du biochar du bois de pin. Nous avons observé une efficacité d'élimination d'environ 99,6 % pour le microbiochar du lisier de porc à une concentration de 500 µg L^{-1} (DCF) en mode batch. La diffusion

intraparticule s'est avérée être le principal mécanisme expliquant l'adsorption pour les deux types de biochar.

4. *La fonctionnalisation de microbiochar a amélioré l'efficacité d'élimination de DCF* :La fonctionnalisation avec de l'acide citrique permis d'augmenter au total le nombre de groupes fonctionnels acides par rapport aux contrôles de 23,6%, 10,2% et 26,2% pour le bois de pin, fumier de porc et coquille d'amande microbiochars, respectivement. La fonctionnalisation avec de l'acide citrique améliore la capacité d'adsorption du *microbiochar*. Pour le biochar de pin, l'élimination du DCF en équilibre est passée de 65 à 80 %. Pour le biochar à base de coquilles d'amandes, dans des conditions similaires, l'efficacité d'élimination de DCF est passée de 58 à 84 %.

5. Les résidus de l'industrie du jus de pomme, le marc de pommes (49,1±2,5 U/gds) et les déchets solides de pâtes et papiers (52,4±2,2 U/gds) se sont avérés être des substrats efficaces pour la production de laccases comparés aux fibres d'alfa séchées (14,2±0,8 U/gds). Le sulfate de cuivre est un inducteur efficace pour la production de laccases en comparaison avec le twen-80, le vératryle alcool de et le rouge de phénol. La dégradation de DCF (500 µg L^{-1}) a été étudiée en utilisant la laccase brute dans les conditions expérimentales suivantes : pH de 4,5 et température de 50 °C. Dans ces conditions, nous avons observé jusqu' à 99 % de dégradation du DCF dans les 5 heures. Les produits de transformation initiaux 3-hydroxydiclofénac, le 4-hydroxydiclofénac et le 5-hydroxydiclofénac ont été identifiés par LC-MS/MS. Le mécanisme de dégradation de DCF catalysé par laccase a été proposé pour l'ouverture du cycle d'hydroxylation et la minéralisation finale en CO_2, NH_3, H_2O et chlore.

6. *En testant tous les* biochars, *l'activité initiale de la laccase a augmenté dans la solution brute, l'efficacité de l'immobilisation par adsorption a également augmenté*. Le microbiochar du lisier de porc a été efficace (31,4 ± 3,1 U g^{-1}) à 10 U mL^{-1} d'activité enzymatique (laccase) suivie coquille d'amande microbiocharbon (24,3 ± 4,8 U g^{-1}) et de bois de pin microbiocharbon (14,58 ± 3,3 U g^{-1}). L'adsorption monocouche homogène est un mécanisme clé avant l'utilisation de laccase

immobilisée sur la liaison de biocharbon qui dégrade totalement (100%) le DCF dans les premières 5 heures à une concentration d'environs (500 ug L^{-1}).

7. La laccase immobilisée sur le biochar par la méthode covalente présentant une stabilité améliorée. Avec l'utilisation du glutaraldéhyde (5 % *v/v*), le microbiochar du lisier de porc a avait la plus grande capacité d'immobilisation des laccases la plus élevée (34,1±1,1 U g^{-1}), suivie du microbiochar des coquilles d'amande (25,3±2,8 U g^{-1}) et du microbiochar du bois de pin (16,18±0,3 U g^{-1}). Le prétraitement à l'acide citrique a permis d'améliorer la capacité d'immobilisation des laccases pour tous les types de microbiochars, particulièrement pour le microbiochar de coquilles d'amande. Plus de 40 % de l'activité de laccase a été conservée pour tous les systèmes microbiochars-laccases après 5 cycles de traitement de DCF (chaque cycle a duré 6 heures).

8. Le biochar lié à la laccase a montré une efficacité d'élimination plus élevée que le biochar brut, une amélioration significative du temps de résidence a été observée qui a été attribuée à la biodégradation du DCF dans la colonne. Le microsystème imprégné de biochar et d'enzymes (BEMS) est rentable et en même temps permet la valorisation des déchets.

En conclusion et dans cette recherche, « une nouvelle technique hybride et efficace de microsystème immobilisé biocharbon-laccase a été mise en œuvre ». Cette thèse de doctorat a permis d'intégrer les concepts de durabilité (application d'acide organique pour une fonctionnalisation, l'adsorption de contaminants et d'immobilisation par adsorption) et la gestion des déchets et de valorisation ainsi que la rentabilité (biochar à partir de déchets / résiduels en tant que substrats pour la production de la laccase).

RECOMMANDATIONS

Les recommandations suivantes sont tirées pour suite aux différents travaux réalisés dans cette thèse:

1. Un mélange des enzymes immobilisées ligninolytiques (telles que la peroxydase de la lignine et du manganèse) peut être pris en considération pour la dégradation non-sélective et efficace des micropolluants au lieu d'un seul système d'enzyme.

2. La production de biochar doit être optimisée à des fins d'immobilisation spécifique des enzymes, la présence de groupes fonctionnels tels que des amines peuvent améliorer l'immobilisation des enzymes.

3. Le système proposé « microsystème immobilisé biocharbon-enzyme » à l'échelle du laboratoire doit être développée davantage à l'échelle pilote et industrielle (stade tertiaire dans le processus de traitement des eaux usées) après mise à l'échelle et donc avec les adaptations nécessaires.

4. Le système proposé peut être efficace pour le traitement d'autres contaminants émergents, tenant compte des propriétés oxydantes de laccase et sa nature non sélective. En outre, le système proposé peut être utilisé pour le traitement de mélange de contaminants émergents et donc son application peut être très avantageuse en conditions réelles dans une station d'épuration des eaux usées.

5. La laccase immobilisée sur microbiochar peut être utilisé pour la dégradation des nouveaux contaminants dans le sol. Cela servira le double objectif d'assainissement des contaminants dans le sol ainsi que l'amélioration de la fertilité des sols. En outre, la laccase immobilisée sur le microbiochar peut être utilisé pour le traitement de boues d'épuration. La libération progressive de laccase permet la dégradation simultanée des nouveaux contaminants présents dans les boues d'épuration des eaux usées. Ses boues d'épuration peuvent être ainsi utilisées en toute sécurité pour la fertilisation des terres agricoles.

6. Après son application dans les eaux usées, le BEMS peut être soit réutilisé ou appliqué au sol / terres agricoles. Le reste de l'enzyme immobilisée dans la microbiochar sera libéré lentement dans le sol et facilitera la dégradation des contaminants, le biochar permettra d'améliorer la fertilité du sol en mobilisant les éléments nutritifs.

ANNEXES

Chapter 1: part 2

Table A1: transition details in LDTD-APCI-MS/MS

Compound	Diclofenac		Diclofenac-d_4
Parent (m/z)	296		300
Products (m/z)	215	250	219
Collision energy (v)	20	20	20

Table A2: Diclofenac LDTD- APCI-MS/MS method validation parameters

R^2	Intra-day precision (% Relative Standard Deviation (RSD))	Inter-day precision (% Relative Standard Deviation (RSD))	Method Detection Limit (MDL) (ng L^{-1})	
			Limit of Detection (LOD)	Limit of Quantification (LOQ)
0.9995	7.1	9.2	270	1000

Chapter 2: part 1

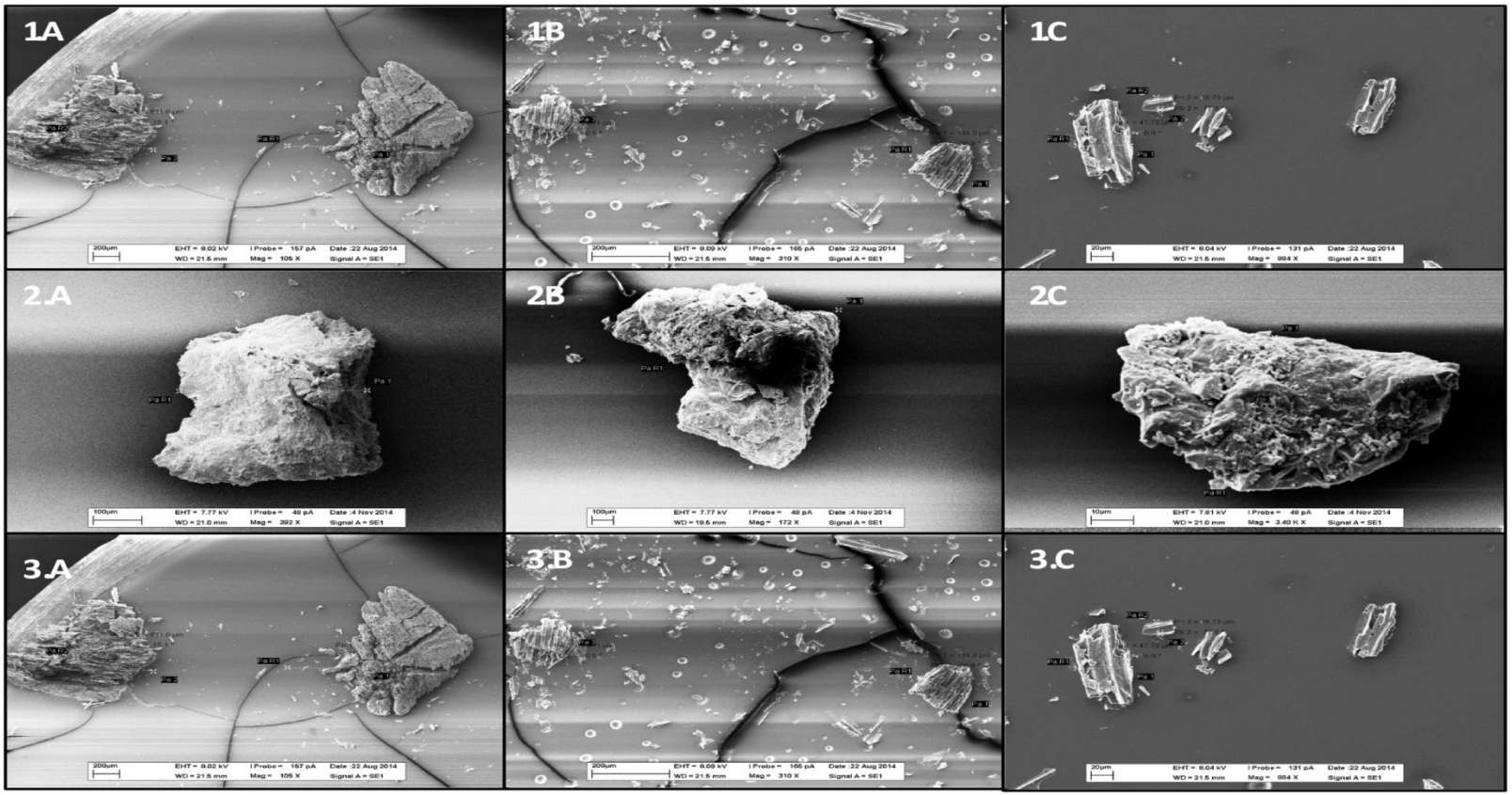


Figure A 1: Scanning electron microscopy images of biochar microparticles: (1.A) Pine wood biochar (BC-PW) - S1; (1.B) BC-PW-S2; (1.C) BC-PW-S3; (2.A) Pig manure biochar (BC-PM)-S1; (2.B) BC-PM-S2; (2.C) BC-PM-S3; (3.A) Paper derived biochar (BC-PD)-S1; (3.B) (BC-PD)-S2; (3.C) (BC-PD)-S3

Chapter 2: part 2

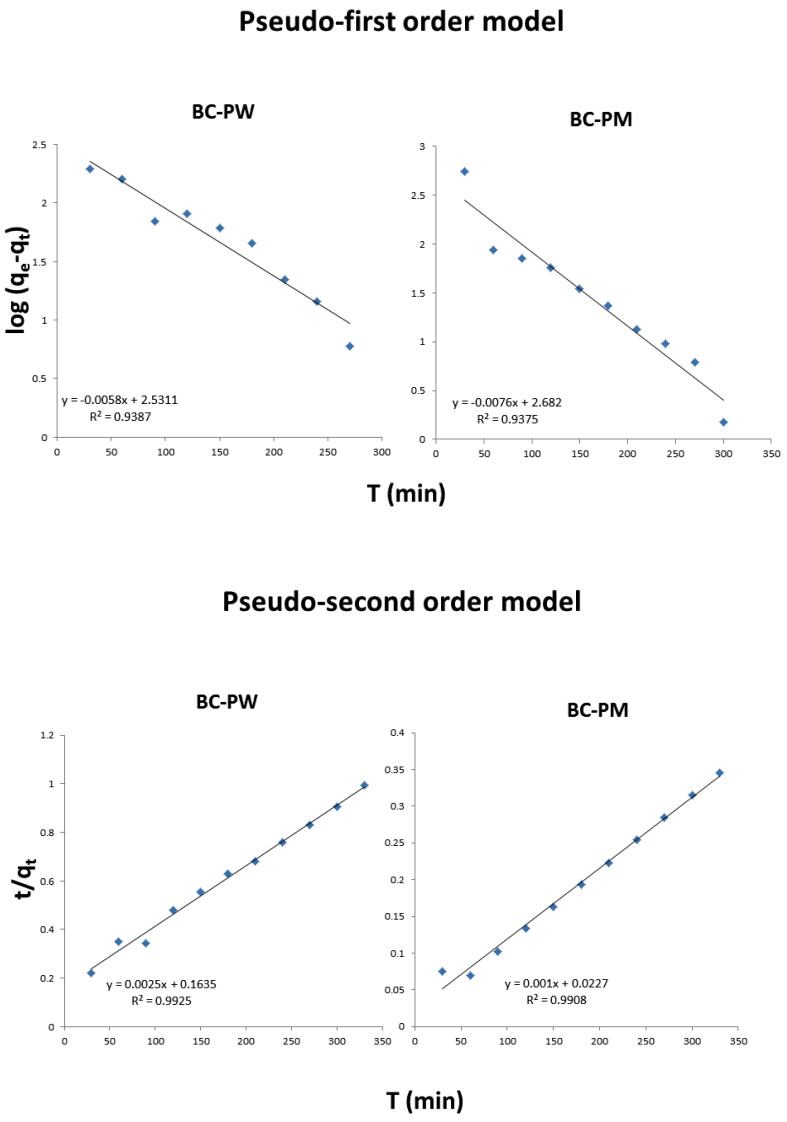


Figure A 2: Pseudo first and second order models for adsorption of diclofenac onto biochars

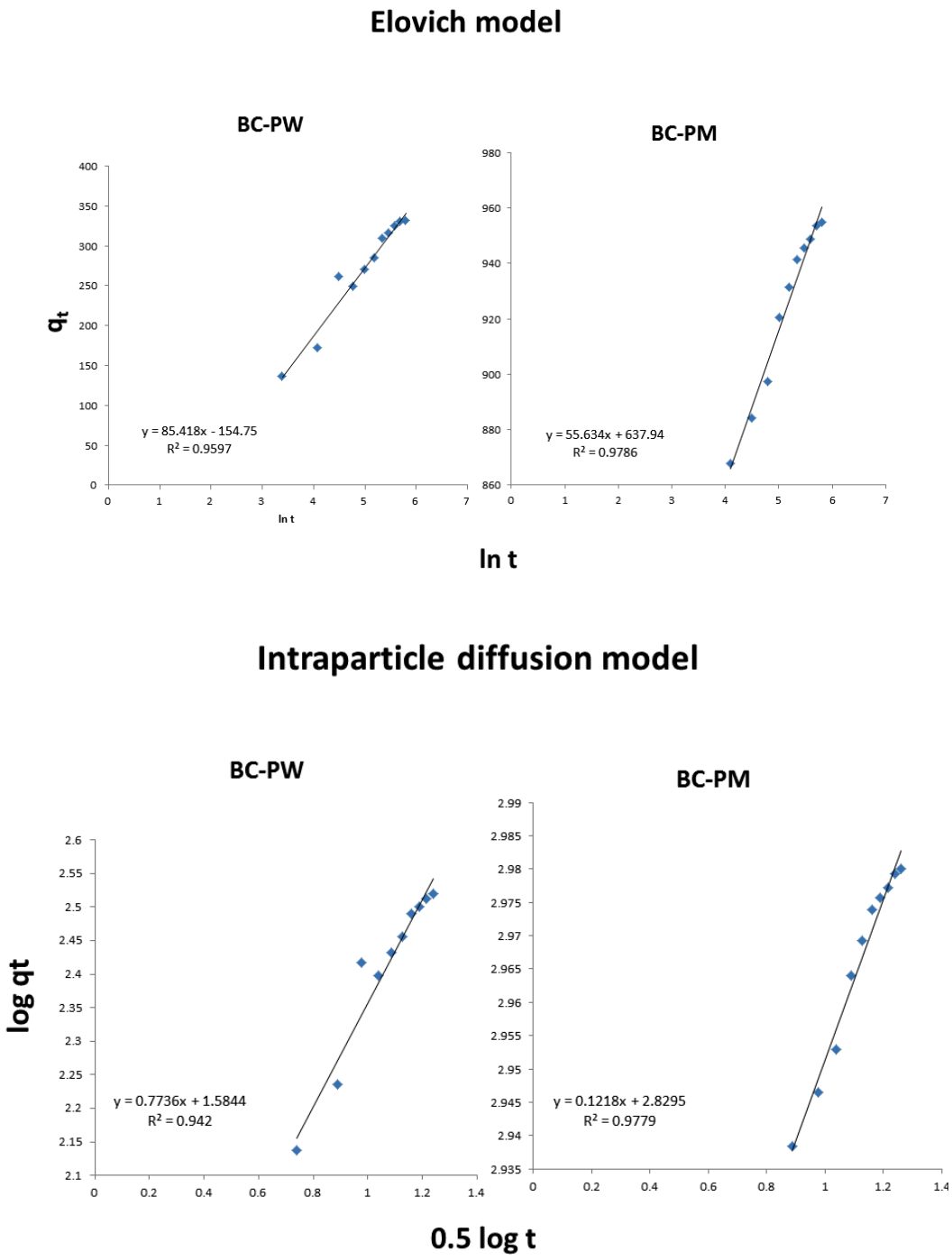
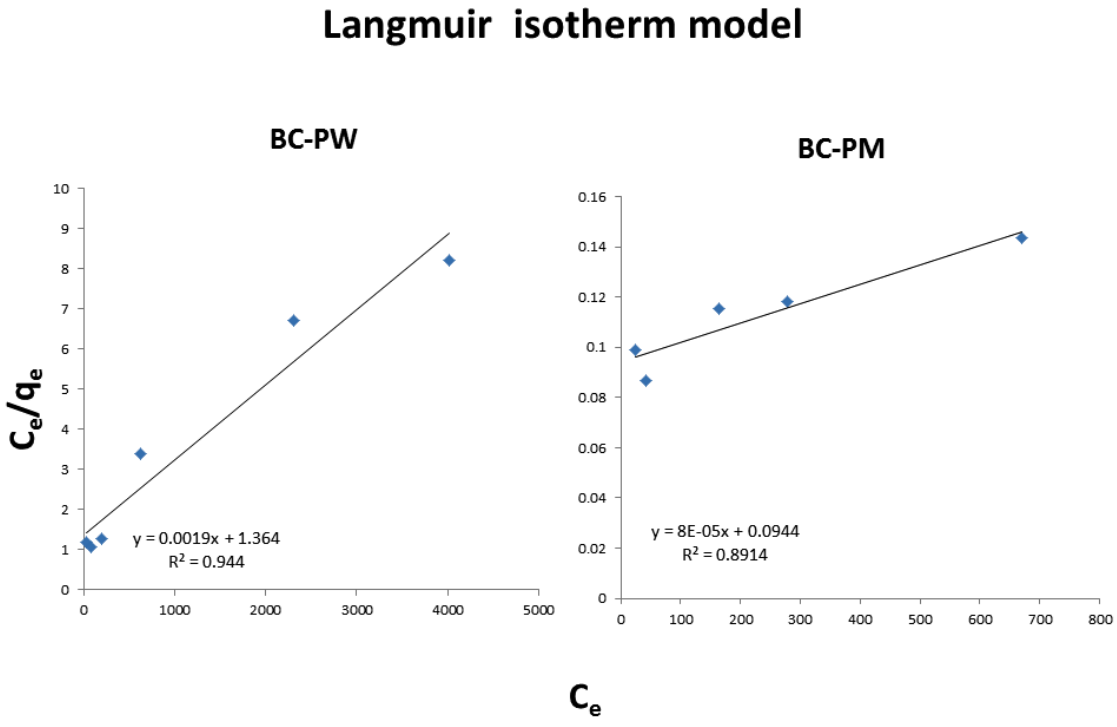


Figure A 3: Elovich and intraparticle diffusion models for adsorption of diclofenac onto biochars



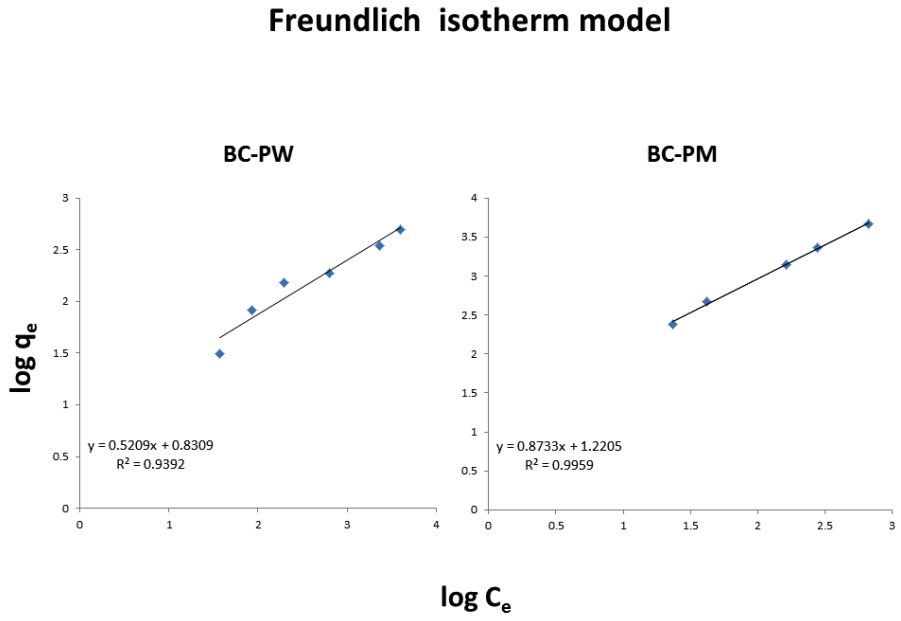


Figure A 4: Langmuir and Freundlich models for adsorption of diclofenac onto biochars

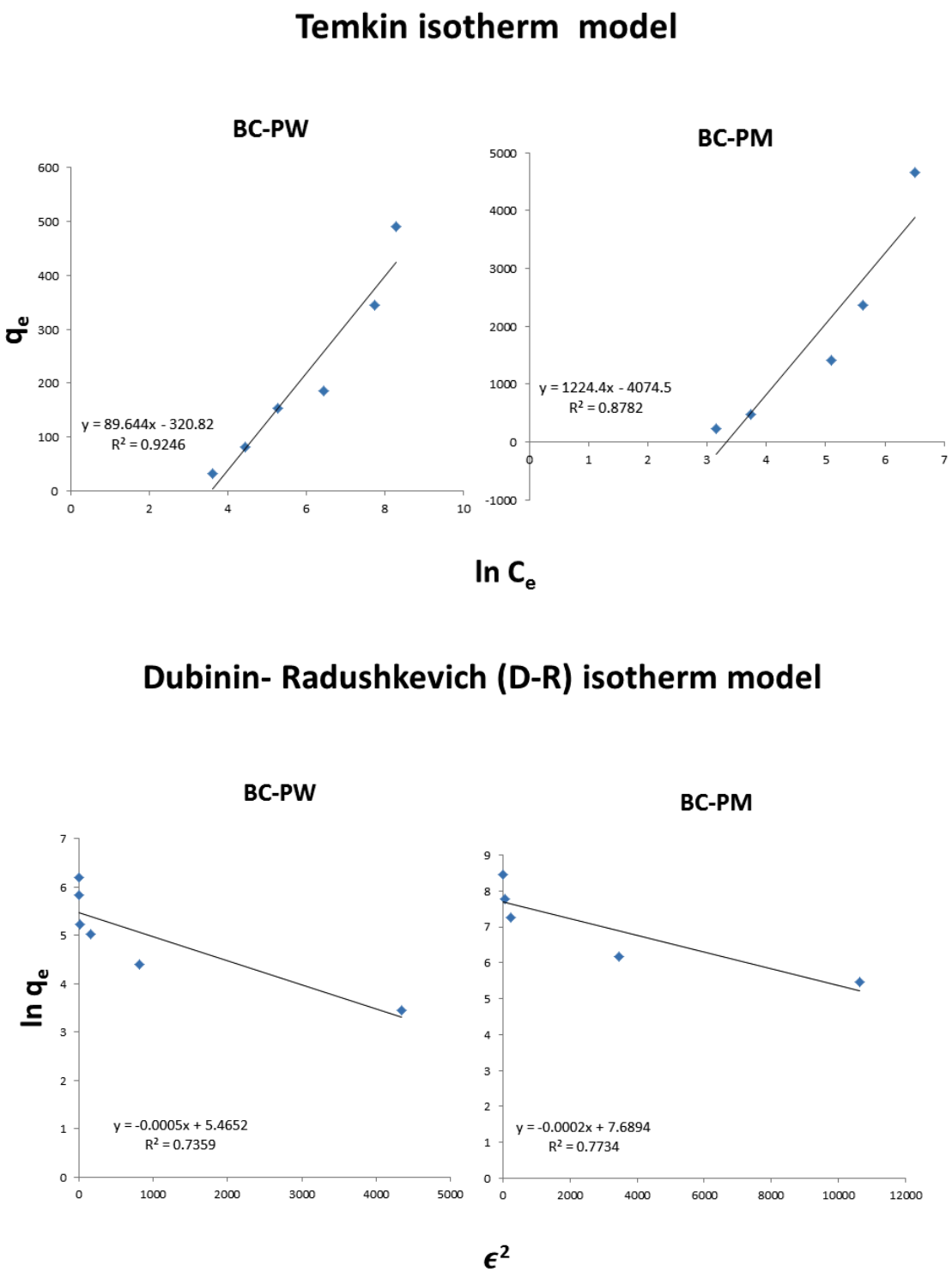


Figure A 5: Temkin and D-R models for adsorption of diclofenac onto biochars

Chapter 4: part 1

Table A 3: Tukey HSD test results: inducers and alfa fibers

Approximate probabilities for post hoc Tukey tests					
Inducers and substrate	Alfa fibers only	Veratryl alcohol and alfa fibers	Copper sulphate and alfa fibers	Tween 80 and alfa fibers	Phenol red and alfa fibers
Alfa fibers only	---	0.972691	*0.012092**	0.284872	0.122968
Veratryl alcohol and alfa fibers	0.972691	---	*0.005449**	0.125999	0.056849
Copper sulphate and alfa fibers	*0.012092**	*0.005449**	---	0.253774	0.776647
Tween 80 and alfa fibers	0.284872	0.125999	0.253774	---	0.912704
Phenol red and alfa fibers	0.122968	0.056849	0.776647	0.912704	---

*Values denoted with * differ significantly at P < 0.05 by Tukey's post-hoc analyses. Each value is mean of three independent replicates.*

Table A 4: Tukey HSD test results: inducers and apple pomace

Approximate probabilities for post hoc Tukey tests

Inducers and substrate	Apple pomace only	Veratryl alcohol and apple pomace	Copper sulphate and apple pomace	Tween 80 and apple pomace	Phenol red and apple pomace
Apple pomace only	---	0.961901	0.198677	*0.040788**	*0.045701**
Veratryl alcohol and apple pomace	0.961901	---	0.078306	*0.016000**	0.106946
Copper sulphate and apple pomace	0.198677	0.078306	---	0.803017	*0.002501**
Tween 80 and apple pomace	*0.040788**	*0.016000**	0.803017	---	*0.000862**
Phenol red and apple pomace	*0.045701**	0.106946	*0.002501**	*0.000862**	---

*Values denoted with * differ significantly at P < 0.05 by Tukey's post-hoc analyses. Each value is mean of three independent replicates.*

Table A 5: Tukey HSD test results: inducers and Pulp and paper solid waste (PPSW)

Approximate probabilities for post hoc Tukey tests

Inducers and substrate	PPSW only	Veratryl alcohol and PPSW	Copper sulphate and PPSW	Tween 80 and PPSW	Phenol red and PPSW
PPSW only		0.999994	0.060881	*0.047455**	0.455423
Veratryl alcohol and PPSW	0.999994		0.057161	*0.044641**	0.484642
Copper sulphate and PPSW	0.060881	0.057161		0.998553	*0.012954**
Tween 80 and PPSW	*0.047455**	*0.044641**	0.998553		*0.010640**
Phenol red and PPSW	0.455423	0.484642	*0.012954**	*0.010640**	

*Values denoted with * differ significantly at P < 0.05 by Tukey's post-hoc analyses. Each value is mean of three independent replicates.*

Table A 6: Univariate tests of significance for laccase activity for alfa fibers and inducers

Variable	Sum of Squares (SS)	Degree of Freedom	Mean Square (MS)	F	p
Intercept	1840.186	1	1840.186	704.5021	0.000000
Inducers for alfa fibers	86.984	4	21.746	8.3253	0.004293
Error	23.508	9	2.612		

Table A 7: Univariate tests of significance for laccase activity for apple pomace and inducers

Variable	Sum of Squares (SS)	Degree of Freedom	Mean Square (MS)	F	p
Intercept	23319.52	1	23319.52	2042.387	0.000000
Inducers for apple pomace	609.63	4	152.41	13.348	0.000798
Error	102.76	9	11.42		

Table A 8: Univariate tests of significance for laccase activity for pulp and paper solid waste and inducers

Variable	Sum of Squares (SS)	Degree of Freedom	Mean Square (MS)	F	p
Intercept	15539.36	1	15539.36	760.3917	0.000001
Inducers for pulp and paper solid waste	1109.70	4	277.42	13.5753	0.006790
Error	102.18	5	20.44		

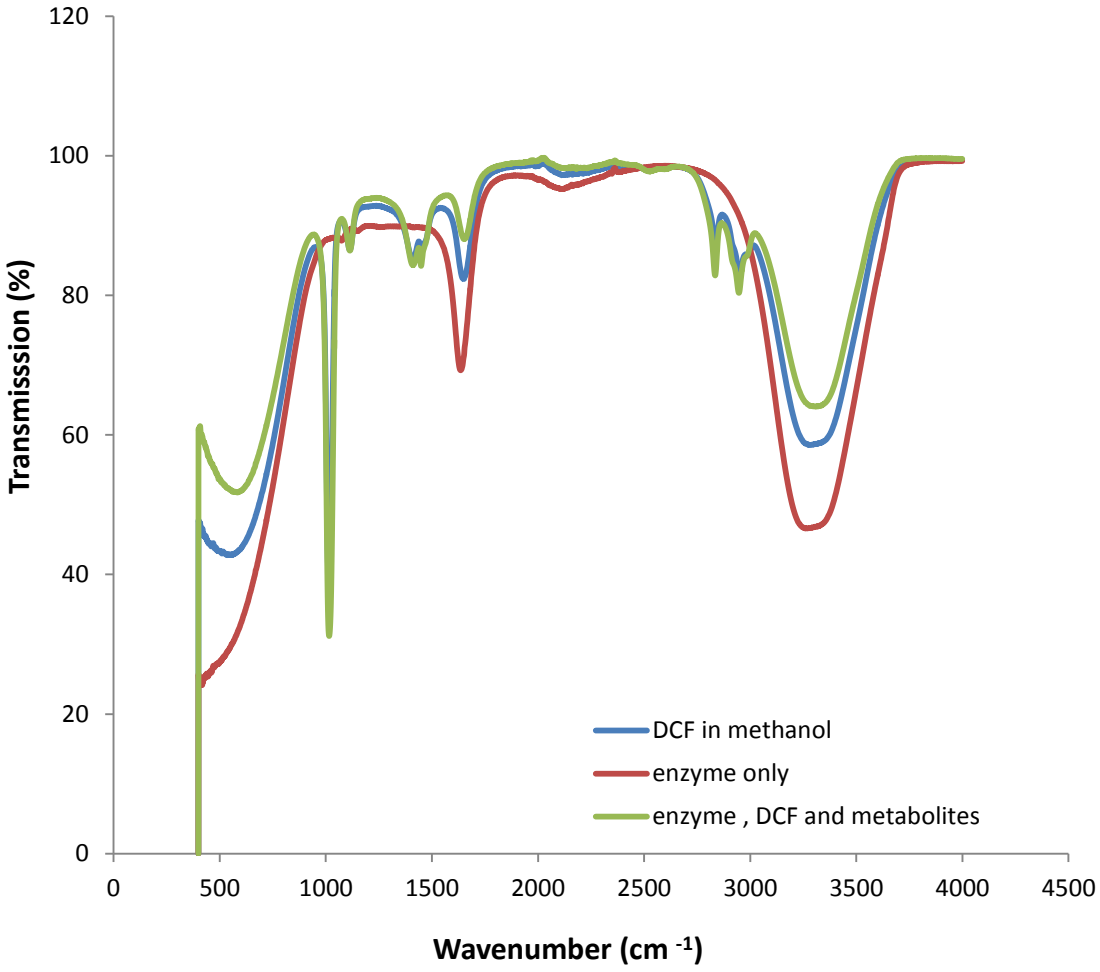


Figure A 6: FTIR spectrum of diclofenac (DCF) and metabolites: a shift around 3200 corresponds to the production of - OH radical at the initial hours of enzymatic treatment

Chapter 4: part 3

Table A 9: Univariate Tests of Significance for loading capacity (the three methods M1. M2 and M3) for each type of biochar

Biochar	*p*
Pig Manure biochar	0.021762
Pine wood biochar	0.018694
Almond shell biochar	0.023044

Table A 10: Tukey HSD test; variable loading capacity- Pine wood biochar

	Pine wood biochar	Method 1	Method 2	Method 3
1	Pine wood method 1		0.955201	*0.023049**
2	Pine wood method 2	0.955201		*0.026675**
3	Pine wood method 3	*0.023049**	*0.026675**	

Table A 11: Tukey HSD test; variable Loading capacity- Pig Manure

	Pig Manure biochar	Method 1	Method 2	Method 3
1	Pig manure biochar method 1		0.967827	*0.030812**
2	Pig manure biochar method 2	0.967827		*0.038258**
3	Pig manure biochar method 3	*0.030812**	*0.038258**	

Table A 12: Tukey HSD test; variable loading capacity-Almond Shell biochar

	Almond Shell biochar	Method 1	Method 2	Method 3
1	Almond Shell biochar method 1		0.861390	*0.039313**
2	Almond Shell biochar method 2	0.861390		*0.033336**
3	Almond Shell biochar method 3	*0.039313**	*0.033336**	