Université du Québec
Institut National de la Recherche Scientifique
Centre Eau Terre Environnement

LE DEVENIR ET LA DEGRADATION DE LA CHLOROTETRACYCLINE DANS LES EAUX USEES ET LES BOUES DES EAUX USEES A L'AIDE DE PROCEDES HYBRIDES AVANCES DE BIO/OXYDATION

Présenté par

Rama Pulicharla

Thèse présentée pour l'obtention du grade de
Philosophiae doctor (Ph.D.)
en sciences de l'eau

Jury d'évaluation

Président du jury et examinateur interne	Antonio Avalos Ramirez Bioprocess Researcher Université de Sherbrooke
Examinateur externe	Trong-On Do, Professor Faculté des Sciences et de Génie Laval University, Canada
Examinateur interne	François Proulx Directeur, Division de la qualité de l'eau Ville de Québec, Canada
Directeur de recherche	Satinder Kaur Brar, Professor INRS-ETE
Codirecteur de recherche	Patrick Drogui, Professor INRS-ETE

DÉDICACE

This thesis is dedicated to my parents and my almighty for their love, motivation and endless support

REMERCIEMENTS

Firstly, I would like to express my sincere gratitude to my supervisor Dr. Satinder Kaur Brar and co-supervisor Dr. Patrick Drogui, for their patient guidance, continuous encouragement, support and their valuable suggestions helped me to develop this Ph.D. Project successfully and to prepare my doctoral examination.

I would also want to thank all of my examiners who took time out of their busy schedules and their valuable comments and suggestions are very helpful in orientation and improving the quality of my Ph.D. project. Very special thanks to application specialist Serge Auger and director Jean Lacoursière from Phytronix for their support and assistance in sample analysis. I also want to thank Denis Dufour, a technician from wastewater treatment plant, Quebec, who guided and helped me in wastewater sampling and also gave information about plant capacity and related information in detail.

With a special mention to Roggy Drouinaud and Dr. Francois Proulx, the Division de la Qualité de l'eau team. It was fantastic to have the opportunity to do part of my research work in their facilities.

I would like to express my gratitude and appreciation to Prof. Jean-François Blais and Dr. Lucie Coudert for assisting and providing necessary information to do cost calculations in my thesis.

A very special gratitude goes to NSERC for providing the funding for my work. Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) support for the doctoral research fellowship is also appreciated.

My sincere thanks also go to all laboratory personnel from INRS, especially Stefane Prémont, Stephane Moisë, Sebastien Duval and Anissa Bensadoune for all the sample analysis and timely help that they have provided.

I also so grateful to have good team members and working with them was a great experience and they helped in my work and they have given encouragement and support throughout my Ph.D. I would like to say big thanks to my colleagues and friends specifically Dr. Mausam Verma, Dr. Tarek Rouissi, Dr. Saurabh Jyoti Sharma, Dr. Ratul Kumar Das, Dr. Vinayak Pachapur, Dr, Dipti Prakash Mohapatra, Dr. Mehdi Zolfaghari, Linson Lonappan, Taheran Mehrdad, Aracel Dalila, Sruthi Chandrika Dokku, Divya Dokku, Gayatri Suresh, Preetika kuknur, Tayssir Guedri, Mitra Naghdi, Mona Chaali, Dr. Hegde Krishnamoorthy, Pratik Kumar, Fatima Ezzahra Bendourou, Agnieszka Cuprys, Amine Mohamed Laadila, Alexandre Mateos, Shravan Kumar, Anay Reddy.

Finally, I must express my very profound gratitude to my parents Srinivas Reddy and Lakshmi Kumari and my sister Manjula Reddy and my almighty for being with me and for their unfailing support and continuous encouragement.

Thanks for all your encouragement!

RÉSUMÉ

La Chlortétracycline (CTC) est un antibiotique à large spectre. L'ajout de quantités élevées de CTC à l'alimentation du bétail à des fins prophylactiques au cours des 60 dernières années a eu pour effet d'augmenter la concentration de CTC libéré dans l'environnement. L'exposition des bactéries à une concentration élevée de CTC dans l'environnement à amener au développement de bactéries résistantes. Selon la solubilité du CTC, on peut s'attendre à ce que la CTC se retrouve dans les eaux usées et non dans les boues d'épuration. Cependant, la capacité du CTC à former des complexes métalliques stables (à faible solubilité) avec une large gamme de métaux, et la présence de concentrations élevées d'ions métalliques dans les boues crée une forte propriété d'adsorption du CTC, ce qui affecte le cloisonnement du CTC dans la station d'épuration. Les procédés de traitement des eaux usées conventionnels ne sont pas efficaces pour dégrader les contaminants organiques à l'état de traces comme la CTC. Des procédés d'oxydation avancée (POA) sont des alternatives efficaces aux traitements conventionnels pour dégrader ou éliminer ces contaminants récalcitrants. Quelques rapports mentionnent que les produits de photodégradation du CTC sont plus toxiques que le CTC. La plupart des études sur la dégradation du CTC ont été réalisées dans les effluents des eaux usées ou dans des solutions aqueuses fortifiées. Comme la CTC se retrouve dans les boues, les procédés de dégradation rapportés pour des solutions aqueuses ne peuvent pas s'appliquer directement à ces dernières.

Dans la présente étude, une nouvelle méthode d'analyse rapide et sensible a été développée pour la quantification du CTC dans les eaux usées et boues d'épuration à l'aide de la source d'ionisation Laser Diode Thermal Desorption (LDTD) couplée à un spectromètre de masse (MS/MS). La concentration des métaux dans les eaux usées et boues d'épuration a été déterminée par spectrométrie à plasma avec couplage inductif d'émission optique (ICP- OES). Des concentrations élevées de CTC ont été détectées dans l'échantillon de boues (0.4-0.8 mg/kg) et non dans les eaux usées (7-64 µg/L), malgré les propriétés hydrophiles du CTC. D'après l'efficacité de distribution et d'élimination de la station d'épuration, -une adsorption élevée de CTC et de ses

complexes métalliques en matière organique a entraîné une accumulation de CTC dans l'échantillon de boues d'épuration. La constante d'équilibre et le ratio molaire du CTC avec les métaux (Ca, Mg, Cu et Cr) ont été déterminés par titration potentiométrique. De plus, ces rapports ont été utilisés pour déterminer la toxicité de complexes CTC-métaux Gram-positif (*Bacillus thuringiensis*) et les bactéries Gram-négatives (*Enterobacter aerogenes*). Les complexes CTC-métaux se sont avérés plus toxiques sur les bactéries à *Bacillus thuringiensis* que le CTC seul cependant les complexes CTC-métaux et la CTC ont montré des toxicités similaires sur la bactérie à *Enterobacter aerogenes*. En outre, l'impact aigu du CTC sur les activités microbiennes (élimination de l'azote et du carbone organique) a été évalué au stade secondaire de traitement d'une station d'épuration.

Même si les stations d'épuration sont efficaces dans l'élimination du CTC des effluents, ils ne dégradent pas le CTC qui s'accumule dans les boues. Par conséquent, les ultrasons (UlS) ont été appliqués pour désintégrer les boues et libérer le CTC adsorbé dans la phase liquide. Les paramètres (fréquence et temps) du procédé UlS ont été optimisés en utilisant l'approche statistique de méthodologie de surface de réponse (MSR) comprenat un plan central composite. L'augmentation de la dégradation de la CTC (82%) a été obtenue par l'oxydation combinée de l'UlS et de l'oxydation de Fenton (FO) par rapport aux POA UlS (67%) et FO (76%).

Le persulfate (PS, $S_2O_8^{2-}$) ayant un potentiel d'oxydation plus élevé (E^0 = 2,01 V), une solubilité et une stabilité supérieures dans l'eau, lui permet d'être un oxydant potentiel dans la dégradation d'une large gamme de contaminants. Le processus d'oxydation lente du PS à température ambiante a été amélioré par une activation homogène (Fe^{2+}) et hétérogène (ZVI, Fe^0) en utilisant un catalyseur ferreux. L'oxydation du PS a entraîné la production d'un radical de sulfate à potentiel d'oxydation plus élevé (E^0 = 2,7 V). Cette nouvelle approche de dégradation de la CTC en utilisant le PS, a entraîné une élimination de CTC de 76 et 94% en imposant un ratio molaire 1: 2 pour 1 mM de CTC en 2h à 500 µM de PS et 1000 µM de fer pour les procédés PS/Fe^{2+} et PS/ZVI respectivement. La génération lente de radicaux sulfates dans un procédé hétérogène (PS/ZVI) est plus efficace qu'avec un temps d'activation de PS par un catalyseur homogène (PS/Fe^{2+}).

En outre, une combinaison de traitement par ultrasons avec une dégradation enzymatique (par la laccase) de CTC a été effectuée pour déterminer l'effet synergique du procédé hybride. Un champignon à pourriture blanche a été utilisé pour produire l'enzyme ligninolytique laccase par fermentation de résidu de pomme à l’état solide. Le CTC a été éliminé à plus de 80% au bout de 2h par un procédé hybride, alors que l’utilisation de la laccase seule a mis un temps de 2 jours pour dégrader 87% de CTC. Le taux de dégradation a augmenté de 14 fois lorsque l'ultrasonication est combinée avec l’action de dégradation de la laccase. Tous les échantillons traités avec des POA hybrides n'ont présenté aucune toxicité œstrogénique.

Enfin, une analyse préliminaire des coûts des POA hybrides a été effectuée pour l'évaluation de procédés efficaces, ce qui justifiera l'adoption de cette technologie dans les conditions de traitement des eaux usées.

ABSTRACT

Chlortetracycline (CTC) is a broad-spectrum tetracycline antibiotic. Adding high amounts of CTC to livestock feed for prophylactic purposes over 60 years led to the constant release of CTC into the environment. The high concentration of CTC exposure to bacteria in the environment results in bacterial resistance development. Based on the solubility of CTC; one can expect that CTC will definitely go to wastewater (WW) and not to wastewater sludge (WWS). However, the ability of CTC to form stable metal complexes (low solubility) with a wide range of metals, the presence of high concentrations of metal ions in sludge and high CTC adsorption property affect the partitioning of CTC in the wastewater treatment plant (WWTP). Conventional wastewater treatment processes are not efficient to degrade trace quantities of emerging contaminants including CTC. Advanced oxidation processes (AOPs) are an alternative to conventional treatment processes to degrade or remove these recalcitrant contaminants effectively. Few reports were said that photo-degraded products of CTC are more toxic than CTC itself. Most of the CTC degradation studies were performed in WW effluents or in spiked aqueous solutions. As CTC goes into the sludge, the reported degradation methods in aqueous solutions are not efficient to extrapolate to real sludge conditions.

In the present study, a new, rapid and sensitive method for quantification of CTC in WW and WWS was developed by using Laser Diode Thermal Desorption coupled with tandem mass spectrometry (LDTD-MS/MS). The metal concentration in WW and WWS sludge was determined using inductively coupled plasma optical emission spectrometry (ICP-OES). High concentrations of CTC were detected in WWS (0.4-0.8 mg/kg) and not in WW (7-64 µg/L), even though CTC is hydrophilic in nature. From overall distribution and removal efficiency of WWTP, it was concluded that high adsorption of CTC and its metal complexes to organic matter resulted in the accumulation of CTC in WWS. The equilibrium constant and the molar ratio of CTC with metals (Ca, Mg, Cu, and Cr) was determined by potentiometric titrations. Further, these ratios were used to determine the toxicity of CTC-metal complexes in Gram-positive (*Bacillus thuringiensis*) and Gram-

negative bacteria (*Enterobacter aerogenes*). CTC-metal complexes were more toxic to *Bacillus thuringiensis* than CTC itself where CTC and its metal complexes were showing similar toxicity in *Enterobacter aerogenes*. Furthermore, the acute impact of CTC on microbial activities (nitrogen and organic carbon removal) was evaluated in secondary treatment stage of a WWTP.

Even though WWTPs are efficient in removing CTC from effluent, but WWTPs not degrading the CTC, as it is accumulating in sludge. Hence, ultrasonication (UlS) AOP was applied to disintegrate the sludge and release the adsorbed CTC into the liquid phase. The UlS process parameters; frequency and time were optimized using response surface methodology (RSM). Increased CTC degradation (82%) was achieved by combined UlS and Fenton oxidation (FO) AOP as compared to alone UlS (67%) and FO (76%) AOPs.

Persulfate (PS, $S_2O_8^{2-}$) having higher oxidation potential (E^0 = 2.01 V), higher solubility and stability in water makes it a potential oxidant in degrading a wide range of contaminants. The slow oxidation process of PS at room temperature was enhanced by homogeneous (Fe^{2+}) and heterogeneous (ZVI) activation using an iron catalyst. PS oxidation resulted in the production of higher oxidation potential sulfate radical (E^0 = 2.7 V). This new approach of CTC degradation by using PS resulted in 76 and 94% CTC removal at 1:2 molar ratio for 1 µM CTC in 2h at 500 µM PS and 1000 µM iron for PS/Fe^{2+} and PS/ZVI processes, respectively. Slow generation of sulfate radicals in heterogeneous (PS/ZVI) process is more efficient than at an activation time of PS by homogeneous (PS/Fe^{2+}) catalyst.

Further, a combination of ultra-sonication treatment with enzymatic (laccase) degradation of CTC was performed to determine the synergistic effect of the hybrid process. *Phanerochaete chrysosporium,* a white-rot" fungus was used to produce the ligninolytic enzyme, laccase by solid state fermentation from apple pomace waste. CTC was removed to over 80% in 2h by a hybrid process where laccase alone took 2 days to degrade 87% of CTC. Degradation rate was increased by 14 folds when ultrasonication was combined with laccase degradation. All treated samples with hybrid AOPs showed no estrogenic toxicity.

Finally, preliminary cost analysis of hybrid AOPs was performed for assessment of efficient process and this will provide rationale in the adoption of the technology under wastewater treatment plant conditions.

PUBLICATIONS DE CETTE THÈSE

1. **Pulicharla, R.,** Mohapatra, D.P., Brar, S.K., Drogui, P., Auger, S., & Surampalli, R.Y. (2014). "A persistent antibiotic partitioning and co-relation with metals in wastewater treatment plant—Chlortetracycline." ***Journal of Environmental Chemical Engineering***, 2 (6): 1596–1603.

2. **Pulicharla, R.,** S Brar, S.K., Drogui, P., Verma, M., & Surampalli, R.Y. (2015). "Removal Processes of Antibiotics in Waters and Wastewaters: Crucial Link to Physical-Chemical Properties and Degradation." ***Journal of Hazardous, Toxic, and Radioactive Waste,*** 19 (4): 04015008.

3. **Pulicharla, R.,** Das, R.K., Brar, S.K., Drogui, P., Sarma, S.J., Verma, M., Surampalli, R.Y., & Valero, J.R. (2015). Toxicity of chlortetracycline and its metal complexes to model microorganisms in wastewater sludge. ***Science of The Total Environment***, 532: 669-675.

4. **Pulicharla, R.,** Brar, S. K., Rouissi, T., Auger, S., Drogui, P., Verma, M., & Surampalli, R. Y. (2017). Degradation of chlortetracycline in wastewater sludge by ultrasonication, Fenton oxidation, and ferro-sonication. ***Ultrasonics Sonochemistry***, 34, 332-342.

5. **Pulicharla, R.,** Zolfaghari, M., Brar, S.K., Drogui, P., Auger, S., Verma, M., & Surampalli, R.Y. "Acute impact of chlortetracycline on nitrifying and denitrifying processes" ***Water Environment Research journal*** (submitted).

6. **Pulicharla, R.,** Das, R.K., Brar, S.K., Drogui, P., Verma, M., & Surampalli, R.Y. "Degradation kinetics of chlortetracycline in wastewater using ultrasonic assisted laccase". ***ACS Sustainable Chemistry & Engineering*** (submitted). .

7. **Pulicharla, R.,** Drouinaud R., Brar, S.K., Drogui, P., Proulx F., Verma, M., & Surampalli, R.Y. “Activation of persulfate by homogenous and heterogeneous iron catalyst to degrade chlortetracycline in aqueous solution”. ***Chemical engineering journal*** (submitted).

8. **Pulicharla, R.,** Rouissi, T., Brar, S. K., Drogui, P., Verma, M., & Surampalli, R. Y. “Techno-economic analysis of hybrid advanced oxidation treatments in the removal of chlortetracycline from wastewater treatment plant” (to be submitted).

PUBLICATION EN DEHORS DE CETTE THÈSE

1. **Pulicharla, Rama,** Mehdi Zolfaghari, Satinder Kaur Brar, Maximiliano Cledon, Patrick Drogui, and R. Y. Surampalli. (2014) "Cosmetic Nanomaterials in Wastewater: Titanium Dioxide and Fullerenes". ***Journal of Hazardous, Toxic, and Radioactive Waste*** 20 (1): B4014005.

2. **Pulicharla, R.,** Marques, C., Das, R. K., Rouissi, T., & Brar, S. K. (2016). “Encapsulation and release studies of strawberry polyphenols in biodegradable chitosan nanoformulation”. ***International journal of biological macromolecules*****,** 88: 171-178.

3. Dhillon, G.S., Kaur, S., **Pulicharla, R.,** Brar, S.K., Cledón, M., Verma, M. and Surampalli, R.Y. (2015). “Triclosan: current status, occurrence, environmental risks and bioaccumulation potential”. ***International journal of environmental research and public health***, 12(5): 5657-5684.

4. Lonappan, L., **Pulicharla, R.,** Rouissi, T., Brar, S. K., Verma, M., Surampalli, R. Y., & Valero, J. R. (2016). « 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”. ***Journal of Chromatography A***, 1433: 106-113.

5. Naghdi, M., Taheran, M., **Pulicharla, R.,** Rouissi, T., Brar, S. K., Verma, M., & Surampalli, R. Y. (2017). “Pine-Wood derived Nanobiochar for Removal of Carbamazepine from Aqueous Media: Adsorption Behavior and Influential Parameters”. ***Arabian Journal of Chemistry*****.**

CONFÉRENCES

1. **R. Pulicharla,** S. Auger, S.K. Brar, P. Drogui, R.Y. Surampalli. **(**2014) **“**A Rapid and Selective MS/MS Method for quantification of light sensitive chlortetracycline drug in wastewater and sludge samples using Laser Diode Thermal Desorption (LDTD) technique”. 62nd ASMS Conference on Mass Spectrometry and Allied Topics. June 15 - 19, Baltimore Convention Center, **USA**.

2. **R. Pulicharla**, S.K. Brar, P. Drogui, R.Y. Surampalli. (2014) “Determination of stability constant of chlortetracycline-metal complexes and its relation with retention of chlortetracycline in wastewater sludge.” 29th Congress of Eastern Canada for Research on Water Quality. October 17, Montreal Polytechnique, Montreal, **Quebec, Canada.**

3. **R. Pulicharla**, S.K. Brar. “Antibiotic-Metal Complexation in Wastewater Treatment Plants: Perplexing Complexity. ” Americana 21-22-23 mars 2017, Place Jean-Paul Riopelle, Montreal, **Quebec, Canada.**

TABLE DES MATIÈRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES ÉQUATIONS

$$O_3 + OH^- \rightarrow O_2 + OH_2^-$$

$$O_3 + HO_2^- \rightarrow HO_2^* + O_3^*$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^* + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH^* + H^+$$

$$\mathrm{recovery} = \left(\frac{\mathrm{C_m}}{\mathrm{C_o} + \mathrm{C_S}}\right) * 100$$

$$\mathrm{m} = \mathrm{Q} \times \mathrm{S}$$

$$\text{Sludge retention time (SRT)} = \frac{\mathrm{C_{MLSS}\,V_r}}{\mathrm{Q_w\,C_{solid}}}$$

$$\mathrm{y} = \beta_0 + \sum_{i=1} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j$$

$$\mathrm{d_{CTC\ sludge}} = [\frac{\mathrm{CTC_{\ sludge}} - \mathrm{B}}{\mathrm{B} - \mathrm{A}}]$$

$$\mathrm{d_{CTC\ sludge}} = 1 \text{ if } \mathrm{CTC_{\ sludge}} \leq \mathrm{A}$$

$$\mathrm{d_{CTC\ sludge}} = 0 \text{ if } \mathrm{CTC_{\ sludge}} > \mathrm{B}$$

$$\mathrm{d_{CTC\ supernatant}} = [\frac{\mathrm{CTC_{\ supernatant}} - \mathrm{A}}{\mathrm{A} - \mathrm{B}}]$$

$$\mathrm{d_{CTC\ supernatant}} = 0 \text{ if } \mathrm{CTC_{\ supernatant}} \leq \mathrm{A}$$

$$\mathrm{d_{CTC\ supernatant}} = 1 \text{ if } \mathrm{CTC_{\ supernatant}} > \mathrm{B}$$

$$\mathrm{d_{TDS}} = [\frac{\mathrm{CTC_{\ TDS}} - \mathrm{A}}{\mathrm{A} - \mathrm{B}}]$$

$$\mathrm{d_{TDS}} = 0 \text{ if } \mathrm{CTC_{\ TDS}} \leq \mathrm{A}$$

$$\mathrm{d_{TDS}} = 1 \text{ if } \mathrm{CTC_{\ TDS}} > \mathrm{B}$$

$$\mathrm{d_{Energy}} = [\frac{\mathrm{CTC_{\ Energy}} - \mathrm{B}}{\mathrm{B} - \mathrm{A}}]$$

$$\mathrm{d_{Energy}} = 1 \text{ if } \mathrm{CTC_{\ Energy}} \leq \mathrm{A}$$

$$\mathrm{d_{Energy}} = 0 \text{ if } \mathrm{CTC_{\ Energy}} > \mathrm{B}$$

$$\mathrm{D} = (\mathrm{d_{CTC\ sludge}} \times \mathrm{d_{CTC\ supernatant}} \times \mathrm{d_{TDS}} \times \mathrm{d_{Energy}})^{1/4}$$

$$\text{Ultrasonic intensity} = \frac{\frac{\text{Joules}}{\text{time}}}{\text{UlS probe diameter}}$$

$$\eta = \frac{P_{Cal}}{P_{Elec}}$$

$$P_{Cal} = P_{UlS} = \left(\frac{dT}{dt}\right) t_{US}.\, C_p.\, M_{sludge}$$

$$E \propto A^2$$

$$S_2O_8^{2-} + Fe^{2+} \rightarrow Fe^{3+} + SO_4^{-*}$$

$$S_2O_8^{2-} + Fe^{2+} \rightarrow Fe^{3+} + SO_4^{2-} + SO_4^{-*}$$

$$SO_4^{-*} + Fe^{2+} \rightarrow Fe^{3+} + SO_4^{2-}$$

$$SO_4^{-*} + H_2O \rightarrow OH^* + H^+ + SO_4^{2-}$$

$$SO_4^{*-} + SO_4^{-*} \rightarrow 2SO_4^{2-}$$

$$SO_4^{-*} + OH^* \rightarrow HSO_4^-$$

$$S_2O_8^{2-} + Fe^0 \rightarrow Fe^{2+} + SO_4^{2-}$$

$$Fe^{3+} + Fe^0 \rightarrow Fe^{2+}$$

$$Fe^0 + H_2O \rightarrow Fe^{2+} + H_2 + OH^-$$

$$C_{22}H_{23}ClN_2O_8 + 48N_2S_2O_8 + 36H_2O \rightarrow 22CO_2 + 48Na_2SO_4 + 48H_2SO_4 + HCl + N_2$$

$$S_2O_8^{2-} + 2I^- \rightarrow 2SO_4^{2-} + I_2$$

$$\%\ \text{recovery} = (C_f/C_i) \times 100$$

LISTE DES ABRÉVIATIONS

AC	activated carbon;
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
AOP	advanced oxidation process
BOD	biological oxygen demand
CCAR	Canadians on Controlling Antimicrobial Resistance
CFU	colony forming units
CTC	chlortetracycline
COD	chemical oxygen demand
CVMA	Canadian Veterinary Medical Association
DOC	dissolved organic carbon
DOM	dissolved organic matter
EDTA	ethylene diamine tetra acetic acid
FAO	Food and Agriculture Organization
FDA	food and drug administration
FO	fenton oxidation
FO	L'oxydation de Fenton
FS	ferrosonication
GM	Growth media
LC	liquid chromatography
LDTD	laser diode thermal desorption
LOD	limit of Quantification
LOQ	limit of Detection
ICP-OES	inductively coupled plasma- optical emission spectrometry

IDL	instrument Detection Limit
MDL	method Detection Limit
MRM	multiple reaction monitoring
NOM	natural organic matter
OIE	World Organization for Animal Health
POA	procédés d'oxydation avancée
PS	persulfate
RSM	response surface methodology
SPE	solid-phase extraction
SS	Synthetic sewage
SS1	Secondary sludge 1
SS2	Settled secondary sludge 2
STEU	station d'épuration des eaux usées
TC	tetracycline
TOC	total organic carbon
TPs	transformation products
UAL	ultrasonic assisted laccase
UlS	ultrasonication
UPLC-MS/MS	ultra-pressure liquid chromatography-mass spectrometry
UV	ultra violet
UV	ultraviolets
WHO	World Health Organization
WW	wastewater
WWS	wastewater sludge
WWTP	wastewater treatment plant
YES	yeast estrogen screen

ZVI	Zero valent iron

CHAPITRE 1

SYNTHÈSE

PARTIE 1. INTRODUCTION

Les composés résiduels pharmaceutiquement actifs dans l'environnement sont considérés comme une classe de contaminants émergents. Des quantités considérables de produits pharmaceutiques inchangés et de leurs métabolites sont constamment détectées dans l'environnement à l'état de trace, et leur risque potentiel pour l'environnement et la santé humaine n'est pas encore connu. Parmi ces composés pharmaceutiques, les antibiotiques sont le troisième groupe le plus important de composés utilisés à travers le monde (Pulicharla *et al.*, 2015). Depuis l'introduction des antibiotiques dans les années 1940, leur rôle a été élargi du traitement à la prévention et la protection des humains contre les infections. Indépendamment des humains, l'utilisation prophylactique des antibiotiques à des niveaux sous-thérapeutiques a été autorisée au cours des 50 dernières années pour la promotion de la croissance, la prévention des maladies et pour l'amélioration de l'efficacité de l'alimentation. Van Boeckel *et al.*, 2015 a calculé la consommation totale d'antibiotiques dans le bétail, soit 63 151 tonnes en 2010 et selon le projet la consommation d'antimicrobiens augmentera de 67% (105 596 tonnes) d'ici 2030. Cette énorme consommation d'antibiotiques conduit à une augmentation potentielle de la pression de sélection sur les bactéries ce qui est considéré comme un des principaux facteurs de résistance aux antibiotiques.

Les tétracyclines (TCs) sont de anciens antibiotiques à large spectre très utilisés en médecine humaine, dans l'industrie de l'alimentation du bétail et dans l'aquaculture afin de favoriser la croissance, et aussi utilisées comme mesures préventives en raison de leurs valeurs thérapeutiques importantes (Sarmah *et al.*, 2006). Selon la Food and Drug Administration (FDA) des États-Unis, les CTC ont contribué à 42% des ventes totales d'antimicrobiens activement commercialisés et approuvés pour l'utilisation dans la production animale de denrées alimentaires en 2011, ce qui les désignent comme le groupe pharmaceutique best-seller (Food *et al.*, 2010). La chlortétracycline (CTC) a été le premier membre de la famille des TCs découverte à la fin des années 1940. Actuellement, la CTC est largement utilisée à des niveaux thérapeutiques et sous-thérapeutiques chez les bovins de boucherie, les poulets et les porcs dans la plupart des pays (Directorate, 2002). CTC est excrété jusqu'à taux de 30-90% dans l'urine et les excréments du bétail et des humains (Eisner *et al.*, 1963, Wouters *et al.*, 2005).

La littérature a montré l'omniprésence des CTC en tant que micropolluants dans divers environnements, compris les déchets animaux, les sols, les eaux de surface, les ruissellements agricoles, les effluents des hôpitaux, les effluents d'eaux usées et les boues d'épuration. Tout en considérant la structure des CTC, le système à quatre anneaux avec de multiples groupes fonctionnels ionisables contenant O- et N-, forme des complexes forts avec une large gamme d'ions métalliques. Une fois que la CTC se retrouve dans les eaux usées municipales, les propriétés physico-chimiques changent en fonction de sa chélation avec les métaux, sa capacité d'adsorption et la composition de des eaux usées. Même les ions métalliques après complexation avec CTC peuvent affecter de nombreux facteurs, tels que l'activité antibactérienne, la dégradation et la stabilité de la CTC.

Les procédés traditionnels de traitement des boues, comme le compostage et la stabilisation, ont montré peu ou pas de capacité de biodégradation de la CTC. Même les produits photodégradés de CTC se sont avérés plus toxiques que le composé d’origine. Les procédés d'oxydation avancée (POA) sont une alternative aux procédés de traitement conventionnels pour dégrader ou éliminer efficacement ces contaminants récalcitrants, compris le CTC. Les POA impliquent la génération des radicaux hydroxyles ou sulfate puissants pour dégrader les polluants organiques récalcitrants ou les transformer en composés plus biodégradables. Le POA autonome peut ne pas être efficace pour la minéralisation complète des contaminants organiques à des concentrations minuscules et peut également conduire à la formation de sous-produits toxiques. Les recherches actuelles sont donc axées sur le devenir et la dégradation de la CTC en utilisant des technologies hybrides POA dans une station de traitement des eaux usées. Ainsi, une meilleure élucidation de la nature des produits de dégradation est nécessaire pour évaluer l'efficacité du traitement. Sur la base des propriétés de la CTC et de l'efficacité de l'POA, cette thèse de doctorat a été divisée comme suit: 1) distribution de CTC à différents stades de traitement de la station d'épuration; 2) la stabilité des complexes CTC-métal et leur toxicité; 3) l'effet du CTC sur l'élimination biologique de l'azote dans la station d'épuration des eaux usées (STEU); 4) différents

POA hybrides pour dégrader la CTC dans les eaux usées et les boues d'épuration et évaluation de la toxicité; et 5) évaluation préliminaire des coûts des POA hybrides étudiés.

PARTIE 2. REVUE DE LA LITTÉRATURE

2.1 Contaminants émergents – antibiotiques

Les contaminants émergents sont des produits chimiques qui ont été largement détectés dans l'environnement à des niveaux de traces et dont les risques potentiels pour l'environnement et la santé humaine font encore l'objet de nombreuses investigations. Ils comprennent les produits pharmaceutiques, les composés perturbateurs endocriniens, les produits de soins personnels, les herbicides et les pesticides. Les produits pharmaceutiques sont conçus pour avoir des effets biologiques spécifiques à des fins thérapeutiques. De 1985 à 2012, selon les données publiées sur les ventes (R. Daghrir *et al.*, 2013b, Organization, 1988), les agents antimicrobiens font partie des trois produits pharmaceutiques les plus vendus (Pulicharla *et al.*, 2015a), et en 2009, 41% des ventes d'agents antimicrobiens était occupé par le marché d'antibiotiques (Hamad, 2010). Depuis l'introduction des antibiotiques dans les années 1940, leur rôle a évolué du traitement à la prévention, la protection des humains contre les infections. En dehors des humains, l'utilisation prophylactique des antibiotiques chez le bétail à des niveaux sous-thérapeutiques a été autorisée au cours des 50 dernières années pour la promotion de la croissance, la prévention des maladies et pour améliorer l'efficacité de l'alimentation.

Les demandes de protéines animales augmentent globalement à un rythme sans précédent par rapport à la population, ce qui se traduit par l'utilisation régulière et intensive des antibiotiques par les agriculteurs pour optimiser la production et répondre aux besoins actuels. Entre 2000 et 2010, la consommation d'antibiotiques chez l'homme a augmenté de 36% dans le monde. *Van Boeckel et al.* 2015 ont estimé à 63 151 tonnes la consommation totale d'antibiotiques en 2010. Il est prévu une augmentation de consommation d'antibiotique de 67% (105 596 tonnes) en 2030. Au cours des deux dernières décennies, la présence de résidus d'antibiotiques dans l'environnement a attiré une grande attention en tant que polluants émergents.

2.1.1 Tétracyclines (TCs) - Chlortétracycline (CTC)

Les TCs sont des antibiotiques à large spectre actifs contre un grand nombre de bactéries Gram-positives et Gram-négatives. En 2011, les TCs ont contribué à 42% des ventes totales d'antimicrobiens qui ont été activement commercialisées aux États-Unis et approuvées pour l'utilisation chez les animaux producteurs de denrées alimentaires, ce qui indique qu'ils représentent le groupe pharmaceutique le plus vendu (Food *et al.*, 2010). Les TCs étaient également les composés antibactériens les plus utilisés dans d'autres pays, dont le Royaume-Uni, le Canada, le Kenya, la Nouvelle-Zélande et la Chine (Sarmah *et al*., 2006). Parmi les TCs, la CTC est l'antibiotique le plus couramment utilisé à des fins vétérinaires à travers le monde.

Chlorotétracycline (IUPAC : 2-[amino(hydroxy)méthylène]-7-chloro-4- (diméthylamino)-6,10,11,12a-tétrahydroxy-6-méthyl-4,4a,5,5a-tétrahydrotétracène -1,3,12-trione, $C_{22}H_{23}ClN_2O_8$) est un antibiotique de la famille des tétracyclines à large spectre. Il a été isolé d'un microorganisme du sol, *Streptomyces aureofaciens* en 1945 par Benjamin Minge Duggar. Il s'agit du premier antibiotique à être découvert dans la famille des tétracyclines. La CTC montre sa propriété antibactérienne (bactériostatique) en inhibant le transfert de l'ARN-t aminoacyle au complexe ribosome ARNm au site accepteur (sous-unité ribosomale 30S), inhibant ainsi la synthèse protéique bactérienne et la croissance cellulaire (Chopra *et al*., 2001). À la fin de 1948, la CTC avait commencé sa production à grande échelle par fermentation.

2.1.2 Utilisation et propriétés – CTC

En 1951, la FDA a approuvé l'utilisation du CTC comme additif alimentaire dans le bétail (Castanon, 2007). La CTC a une longue histoire d'utilisation réussie dans le traitement des infections humaines et animales en raison de ses propriétés antibactériennes à large spectre, de l'absence d'effets secondaires majeurs et de la rentabilité. La CTC est maintenant largement utilisée à des niveaux thérapeutique et sous-thérapeutique chez les bovins de boucherie, les poulets et les porcs (éleveur, couche) dans la plupart des comtés tel que Australia, European Union, Canada, USA

(Sarmah *et al*., 2006). Un large éventail de doses de CTC est utilisé selon le type de bétail et le but (10 à 500 g/tonne de nourriture).

Table 1.1 Physico-chemical properties of chlortetracycline hydrochloride

Molecular formula	$C_{22}H_{23}ClN_2O_8$ HCl
Molecular weight	515.34 Da
Density	1.7 ± 0.1 g/cm^3 at 25 °C
Water solubility	8.6 mg/mL at 25 °C
Melting point	210-215 °C
Boiling point	821.1 ± 65.0 °C at 760 mmHg
Vapor pressure	0.0 ± 3.1 mmHg at 25 °C
log $K_{o/w}$	-0.53 ± 0.82
pKa*	3.33 ± 0.30 7.55 ± 0.02 9.33 ± 0.30
Storage	-20 °C
Appearance	The yellow crystalline solid
Use	An antimicrobial and antibacterial agent

*pKa values were measured by potentiometric method (Qiang *et al.*, 2004)

Le CTC est un acide organique et sa solubilité est d'environ 8,6 mg/ml dans l'eau à température ambiante. Les propriétés physicochimiques du CTC sont énumérées dans le tableau 1.1. Le CTC montre un comportement amphotère caractérisé par trois valeurs pK_a (constante de dissociation) distinctes, comme le montre la figure 1.1. Il contient trois groupes fonctionnels distincts: le tricarbonyle méthane, la β-dicétone phénolique et le cation ammonium, qui subit une ionisation différente en fonction du pH du milieu environnant. Les valeurs de pK_a de CTC régissent la solubilité, l'absorption, la distribution et l'élimination et aident à la compréhension de phénomènes chimiques, tels que l'absorption biologique, la liaison à des matrices environnementales et la formation de chélates avec des cations métalliques. La CTC existe principalement comme un zwitterion neutre entre pH 4 et 7.

Parmi tous les antibiotiques de la famille des tétracyclines, le CTC a une faible solubilité dans l'eau et présente la plus faible absorption (25-30% de la dose) dans la voie gastro-intestinale. La CTC est excrétée dans l'urine et les selles (circulation entéro-hépatique) dans des quantités inchangées ou microbiologiquement inactives (Agwuh *et al.*, 2006). La demi-vie du CTC est de 6 à 8 heures chez l'homme et le 6-iso-CTC et le 4-epi-6-iso-CTC ont été identifiés comme les métabolites principaux du CTC et du 4-epi-CTC dans le bétail. De petites quantités d'iso-chlortétracycline ont également été observées. Bien que ces métabolites soient microbiologiquement inactifs, leur profil toxicologique n'est toutefois pas connu.

2.1.3 Structure de la CTC - activité antibactérienne

Les molécules de chlorotétracycline comprennent un noyau tétracyclique condensé linéaire (anneaux désignés A, B, C et D sur la figure 1.1. La 6-désoxy-6-déméthyltétracycline est le pharmacophore basique des antibiotiques de la tétracycline qui présente une activité antibactérienne et qui a des configurations stéréochimiques tétracycliques linéaires fusionnées aux positions 4a, 12a (jonction AB) et 4 (diméthylamino) énol (positions 11, 12 et 12a) du cycle D phénolique. Toute modification structurelle de ces groupes fonctionnels entraîne la perte de l'activité antibactérienne de TC (Chopra *et al.*, 2001).

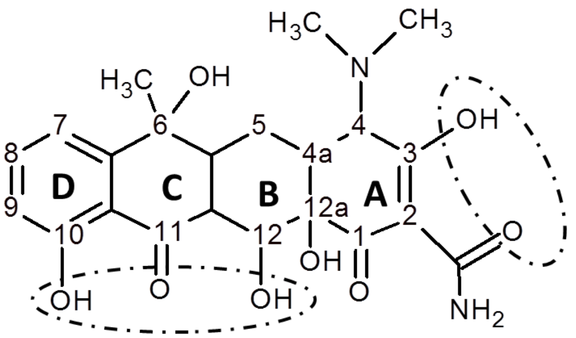


Figure 1.1 **Basic structure of chlortetracycline and metal binding sites**

2.1.4 CTC- complexation et résistance des métaux

La chlorotétracycline a une forte capacité à former des complexes avec une large gamme de métaux. Comme mentionné dans la session 2.1.2, de multiples groupes fonctionnels riches en électrons de CTC, tels que des acétamides (C2), des fragments β-dicétone phénoliques (C10, C11, C12) et des groupes diméthylammonium (C4) (figure 1.1) sont stabilisés par complexation avec des métaux de transition à polarisation élevée. La charge positive des métaux est partiellement partagée avec une charge négative des atomes donneurs d'électrons présents dans la CTC et la délocalisation électronique sur les atomes de partage (anneau chélateur), ce qui stabilise la forte densité électronique de CTC (Ames *et al.*, 2011, Siddappa *et al.*, 2014). De nombreuses études ont été réalisées sur la complexation du CTC avec une large gamme de métaux et les effets du pH de l'environnement environnant sur les rapports d'équilibre molaire du CTC et des métaux. Cette propriété de chélation des métaux peut influencer les propriétés antimicrobiennes et pharmacocinétiques de la CTC. La complexation des métaux semble être la médiation de la liaison des CTC à des sites macromoléculaires (ribosomes, protéines) auxquels ils ne peuvent se lier par eux-mêmes (Orth *et al.*, 1999). Ce CTC liant des métaux a également montré une transportabilité différente à travers la membrane cellulaire Gram-positive et Gram-négative de bactéries due à différentes structures de la paroi cellulaire (Pulicharla *et al.*, 2015b).

La résistance aux TCs a été signalée par trois types de mécanismes: i) l'efflux des TCs à médiation énergétique; ii) la protection des attaques ribosomiques contre les TCs; Et iii) l'inactivation des TCs. La résistance par mécanisme d'efflux pourrait être due à une perméabilité élevée des complexes TC métalliques à travers la membrane cellulaire des bactéries (Chopra *et al.*, 2001) Le répresseur de la tétracycline (TetR) qui régule l'expression des gènes de résistance peut subir des modifications conformationnelles (induction) par le complexe $[\text{Mg-CTC}]^{2+}$ qui a une affinité de liaison plus élevée, conduisant à l'expression des gènes résistants *tet*A et *tet*R, mais pas avec la forme libre (Orth *et al*., 1999).

2.2 CTC dans l'environnement

Une fois que les contaminants émergents entrent dans l'environnement, ils ne peuvent pas disparaître ou être détruits, mais ils sont transformés en différentes formes qui peuvent être toxiques ou non toxiques pour l'environnement. Le sort de ces contaminants émergents dépend des propriétés physicochimiques du composé et des conditions de l'environnement dans lequel ils existent. Les études ont montré un large éventail de niveaux de détection de la CTC dans les eaux usées municipales (0,005-62 µg/L) et les boues d'épuration (15 µg/kg - 33 mg/kg). Le tableau 1.2 présente les concentrations de CTC détectées à travers le monde dans différentes matrices environnementales.

Table 1.2 Detected chlortetracycline concentrations across the world in different environmental matrices.

Country	Source	Concentration	Reference
Ohio, US	Swine lagoon poultry field stream	2-1000 µg/L	(Campagnolo *et al.*, 2002)
Shanghai, China	Huangpu River,	12.91 ng/L	(Jiang *et al.*, 2011)
Jiangsu Province, China	Pond water	0.57 µg/L	(Wei *et al.*, 2011)
	Animal wastewater	0.49-1.10 µg/L	
	Animal form effluent	0.58-3.67 µg/L	
	River water (China)	0.32-2.42 µg/L	
Changping District, Beijing	swine wastewaters	2.65–32.67 µg/L	(Ben *et al.*, 2008)
China	Dung samples of pig	0.16-21.06 mg/kg	(Ling Zhao *et al.*, 2010)
	Dung samples of chicken	0.16-17.68 mg/kg	
	Dung samples of cows	0.24-27.59 mg/kg	

North Zhejiang Province, China	Manure samples	11.3 mg/kg	(Zhang *et al.*, 2008)
Turkey	Animal manure	0.25–0.42 mg/kg	(Karcı *et al.*, 2009)
Austria	Pig manure	0.1-46 mg/kg	(Martínez-Carballo *et al.*, 2007)
	Chicken and turkey manure	1.7 mg/kg	
US	Surface water	0.15 µg/L	(Lindsey *et al.*, 2001)
Wisconsin, USA	Wastewater treatment facilities	0.31-0.42 µg/L	(Karthikeyan *et al.*, 2006)
Chongqing region of Three Gorge Reservoir, China	Hospital effluent	0.017 -0.020 µg/L	(Chang *et al.*, 2010)
	Wastewater effluent	0.157-0.042 µg/L	
	River water	0.015 µg/L	
South–East Queensland, Australia	Wastewater treatment plants	0.005-0.006 µg/L	(Watkinson *et al.*, 2009)
	River water	0.003 µg/L	
Colorado, a western U.S	River water	0.26-0.28 µg/L	(Yang *et al.*, 2003)
Quebec, Canada	Wastewater	62 µg/L	(Puicharla *et al.*, 2014)
	Wastewater sludge	34 mg/kg	
China	Wastewater sludge	15 µg/kg	(Xiangjuan Yuan *et al.*, 2014)
Japan	Organic matter from animal manure	1.3-240 µg/kg	(Motoyama *et al.*, 2011)
Jordan	Table eggs	56-8589 µg/g	(Alaboudi *et al.*, 2013)
Michigan, USA	Wastewater	2.4-8.1 µg/L	(Gao *et al.*, 2012a)

2.2.1 CTC dans les stations de traitement des eaux usées (STEU)

Les stations d'épuration des eaux usées municipales sont une importante source ponctuelle des gènes de résistance aux antibiotiques et aux antibiotiques dans l'environnement. Outre les sources ponctuelles, les antibiotiques se présentent également comme source non ponctuelle de pollution. Environ 70 à 90% de la CTC administrée à des animaux destinés à l'alimentation sont excrétés sans changement dans les selles et l'urine des animaux (Agwuh *et al.*, 2006). Ces déchets animaux lorsqu'ils sont appliqués à des champs comme engrais présentent un risque de propagation d'antibiotiques dans l'environnement par écoulement superficiel (source non ponctuelle). En raison des sources ponctuelles et non ponctuelles de pollution, les antibiotiques sont largement distribués dans l'environnement. La concentration élevée de la CTC non métabolisée provenant des excrétions animales et humaines finit finalement dans les systèmes des eaux usées. Même, les biosolides d'épandage dans les sols sont de plus en plus fréquents en raison de l'élimination sûre et efficace des déchets et de l'utilisation ingénieuse de la valeur des engrais des biosolides. L'enfouissement et l'application de biosolides de déchets municipaux et de fumier comme engrais sur les terres agricoles mènent à l'accumulation de CTC dans le sol. De plus, à partir du sol, le CTC se déversera dans les sources d'eau (eaux de surface et souterraines) et pourra également s'accumuler dans les plantes. Ainsi, la CTC entre dans la chaîne alimentaire. En dehors de cela, la station d'épuration crée une approche appropriée pour que les bactéries interagissent continuellement avec des antibiotiques à des concentrations sous-inhibitrices et provoquent une résistance dans les populations bactériennes (Hirsch *et al.*, 1999, Kemper *et al.*, 2008).

2.2.2 Analyse de la CTC

Le développement de méthodes analytiques sensibles pour détecter les contaminants émergents à des concentrations environnementales dans différentes matrices est nécessaire pour comprendre ce qui adviendra des contaminants. La figure 1.2 montre la représentation schématique des procédures analytiques utilisées pour la détection de CTC en des eaux usées. les boues d'épuration et eau potable. Le méthanol a été utilisé pour l'extraction de CTC par micro-ondes à partir de solides des boues d'épuration. Avant l'extraction en phase solide (nettoyage) des échantillons, le pH a été ajusté en dessous de la valeur de pK_a de 3,0 de la CTC. En dessous des valeurs de pK_a, le CTC était complètement ionisé et absorbé facilement et fermement sur les cartouches pendant le processus de nettoyage. La figure 1.2 montre la procédure d'extraction pour le CTC à partir de différentes matrices. Dans la plupart de la littérature, la chromatographie liquide - spectrométrie de masse (LC-MS/MS) a été utilisée pour détecter la CTC. Récemment, la spectrométrie de masse par désorption thermique à laser de diode (LDTD-MS/MS) et chromatographie liquide à ultra-pression -

spectrométrie de masse (UPLC-MS/MS) ont été utilisées pour la quantification des contaminants. Ces techniques ont montré de limites de detection de respectivement 0,29 ng/L, 0,5 µg/L et 3 µg/L de la limite de quantification pour CTC dans UPLC, LC et LDTD (Puicharla *et al.*, 2014).

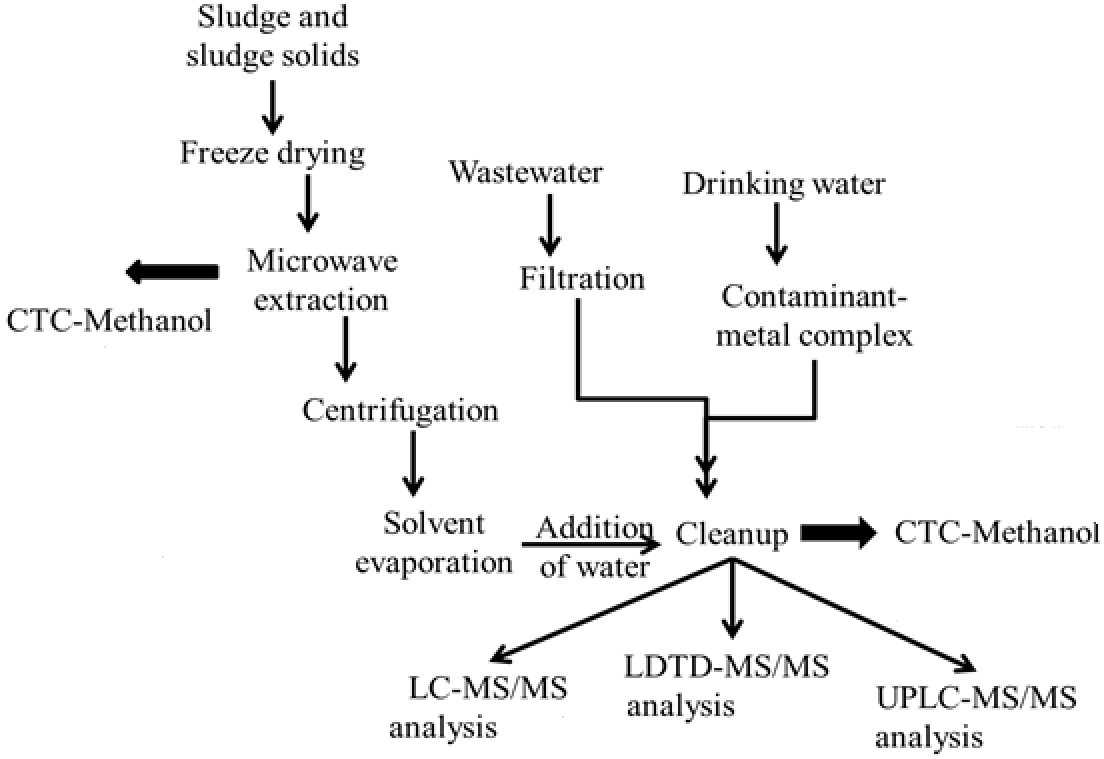


Figure 1.2 Extraction of chlortetracycline from different matrices

2.2.3 Distribution de la CTC dans la STEU

Une fois que la CTC entre dans les eaux usées municipale; les propriétés physicochimiques changent en fonction de sa capacité de chélation et d'adsorption et de sa composition. Cela peut affecter le cloisonnement de la CTC dans les STEU. En se bas ant sur les données de solubilité de CTC, on peut s'attendre à ce que la CTC ira aux eaux usées et non dans les boues d'épuration. Cependant, la présence de concentrations élevées d'ions métalliques et la capacité d'adsorption de CTC ont un effet de cloisonnement dans la STEU et la recherche n'a pas été explorée dans ce domaine. La complexation avec des métaux en les eaux usées modifie les propriétés chimiques du CTC, comme la solubilité. Les complexes CTC-métal ont une faible solubilité par rapport au CTC libre qui affecte le coefficient de partage (log P). Par conséquent, la présence de concentrations élevées d'ions métalliques dans les eaux usées affecte de manière significative la distribution de CTC dans les stations d'épuration entre des eaux usées et les boues d'épuration (Puicharla *et al*., 2014). En dehors de cela, les interactions d'adsorption de CTC avec des substances organiques sont augmentées par le pontage de métal en formant des complexes ternaires, ce qui augmente considérablement l'adsorption de CTC (Gu *et al.*, 2005, Gu *et al.*, 2007, Guerra *et al.*, 2006, Yanping Zhao *et al.*, 2011) Même, les ions métalliques après complexation avec CTC peuvent affecter de nombreux facteurs, tels que l'activité antibactérienne, la dégradation, la stabilité du CTC. Les ions métalliques co-existants et la matrice organique peuvent interagir avec les antibiotiques dans la station d'épuration et cela peut influencer l'élimination de la CTC.

2.2.4 Effet du CTC sur les processus biologiques de la STEU

Le traitement biologique de la STEU, en particulier le procédé des boues activées, contribue de manière significative à la valorisation des eaux usées; Cependant, différents contaminants, en particulier les antibiotiques, affectent négativement la performance des processus biologiques, telles que l'élimination chimique de la demande en oxygène (DCO), la nitrification et la dénitrification. Des concentrations faibles de TC (0,5 mg/L) n'ont pas exercé d'influence sur l'azote biologique, mais une présence de > 5 mg/L de TC a montré une diminution significative de l'efficacité totale d'élimination de l'azote (A Chen *et al.*, 2015).

Outre cela, le processus de traitement biologique crée un environnement potentiellement approprié pour le développement de la résistance et la propagation en raison de l'exposition continuelle des microorganismes à différents antibiotiques à des concentrations sous-inhibiteur. Les études de Droge *et al*., 2000 ont permis de récupérer les plasmides porteurs de gènes TetR et de composants de systèmes d'efflux multidrogue à partir de communautés de boues activées. Des études plus approfondies d'Auerbach *et al*., 2007 ont démontré la présence d'un nombre considérablement plus élevé de types de gènes Tet R dans la STEU que les échantillons naturels d'eau de lac, ce qui implique que les stations d'épuration peuvent être des sources potentielles du développement de la résistance. De plus, la libération de bactéries résistantes aux antibiotiques à travers les effluents de les eaux uses dans les eaux de surface a déjà été signalée (Miller et al., 2016). On a également signalé que les biosolides de la STEU contenaient une concentration plus élevée de bactéries résistantes aux antibiotiques. On a observé des absorptions significativement plus élevées de gènes et de bactéries de résistance aux antibiotiques dans les stations d'épuration opérées par les POA comparativement à celles des stations de traitement classiques. De plus, il n'y a pas de réduction significative des gènes de résistance aux antibiotiques et des bactéries par les processus de désinfection (chloration et UV) (Munir *et al.*, 2011).

2.2.5 Processus d'oxydation avancée pour la dégradation de la CTC

Les usines de traitement des eaux usées contenant des antibiotiques résiduels sont pour la plupart des composés récalcitrants non règlementés et sont difficiles à oxyder et à biodégrader par des traitements biologiques conventionnels et traditionnels. Des études récentes ont démontré que les procédés classiques ont des taux d'élimination variables entre 40-95% pour les résidus de TC (Cirja *et al.*, 2008, Gao *et al.*, 2012b) et génèrent un nouveau déchet. En outre, malgré une élimination de 95%, il existe une possibilité de formation de sous-produits qui peut être plus toxique que l'antibiotique d'origine. Depuis les années 1980, les POA ont été explorés comme des solutions de rechange aux procédés traditionnels de traitement des systèmes d'eau potables. Actuellement, ces technologies avancent et sont largement utilisées pour différentes matrices. Les POA sont les processus d'oxydation impliquant la génération de radicaux hydroxyles ou sulfates puissants pour dégrader les polluants organiques récalcitrants ou les transformer en composés plus biodégradables. De plus, les POA utilisés pour éliminer les contaminants résiduels, y compris les TC dans les boues d'épuration, représentent de façon significative un outil pour la production de biosolides sûrs. Les POA peuvent être réalisés par de multiples procédés incluant l'UV, l'ozonation, l'oxydation de Fenton, l'ultrasonication, les rayons gamma, un faisceau d'électrons, un catalyseur et une enzyme basée sur la génération de radicaux hydroxyles ou sulfates pour dégrader les contaminants récalcitrants selon l'environnement. Le tableau 1.3 énumère les différents POA utilisés jusqu'à présent pour dégrader la CTC dans différentes matrices environnementales.

Table 1.3 Different methods used to degrade chlortetracycline in different environmental matrices.

Matrix	**Degradation method and conditions**	**Degradation efficiency**	**Reference**
Soil–chicken feces mixture	Composting	56% (30 °C), 22% (20 °C), and 1% (4 °C)	(Gavalchin *et al.*, 1994)
Manure of beef calves	Composting, CTC-113 µg/g	75–79% (55 °C, 7 days) 98–99% (55 °C, 30 days) 40–49% (room temperature °C, 30 days)	(Osman A Arikan *et al.*, 2009)
Wastewater	Photocatalysis with Au-TiO_2/nanocube $ZnFe_2O_4$ 5.1 (Cirja *et al.*) and 1 g/L (catalyst dose); CTC-50 mg/L	> 80% at 4 h under visible light irradiation	(Jia *et al.*, 2017)
Wastewater sludge	Ultrasonication combined with fenton oxidation; CTC-33 mg/L	82% removal at 106 min and 60% amplitude	(Pulicharla *et al.*, 2017)
Water	Ozonation; CTC- 0.5 mM	at pH 2.2 and 7.0 complete removal in 8 min	(Khan *et al.*, 2016)
Water	Tris-(2,2′-bipyridine) ruthenium(II) chloride (Ru3B), and tris-(1,10-phenanthroline) ruthenium(II) chloride (Ru3P) as photosensitizers to form singlet oxygen ($^1[O_2]^*$) (indirect photooxidation)	25 mg/L, Ru_3B = 10 mg L, O_2 dose- 97%, pH = 7; 57% removal	(Salazar-Rábago *et al.*, 2016)
Water	N-doped Ti/TiO_2 photoanode under sunlight irradiations; CTC (100 µg/L)	99.6% degraded at 180 min and current intensity of 0.6 A	(Rimeh Daghrir *et al.*, 2013a)
Water	UV-C, E-beam and ozone; CTC-30 mg/L	E-beam and ozone complete removal (90 min); UV-C (no removal)	(Tae-Hun Kim *et al.*, 2012)
Water	Photoelectrocatalytic process with Ti/TiO_2 nanostructured electrodes; CTC-50 mg/L	74.2% removal at current intensity of 0.39 A, 120 min	(Rimeh Daghrir *et al.*, 2012)
Wastewater spiked	photoelectrocatalytic oxidation by N-doped TiO_2	99.1% at 120 min 0.6 A of current	(Rimeh Daghrir *et al.*, 2014)

Animal manure	Anaerobic digesters at 35 °C, 33 days digestion, CTC-5.9 mg/L	75% removal	(Osman A. Arikan, 2008)
Water	Photoelectrocatalytic oxidation using Ti/TiO2 photo-anode, CTC-25 µg/L	98.0 % degradation at 0.39 A under UV irradiation (245 nm), 120 min	(R. Daghrir *et al.*, 2013b)
Spiked municipal wastewater	Electrooxidation–Ultrasonication Hybrid Process with Ti/PbO$_2$, CTC-0.01-100 mg/L	98% removal at 3.92 A, ultrasound irradiation (20 W, at 500 kHz), 95 min	(Tran *et al.*, 2016)
Water	laccase–HBT system, CTC-0.1 mM	48% (laccase, 4h); completely removed (laccase–HBT, 0.25 h) completely removed (1h, MnP)	(Suda *et al.*, 2012)

2.2.5.1 Dégradation des UV (ultraviolets)

La lumière UV a été utilisée dans la photodégradation directe de contaminants dans des environnements aqueux où les composés cibles absorbent l'énergie sous forme de photons de lumière UV et subissent un processus d'oxydation. Des études ont montré que la CTC était très récalcitrante à la dégradation des UV et a signalé une dégradation de 40-50% (Ilho Kim *et al.*, 2009, Tae-Hun Kim *et al.*, 2012). De plus, les produits de dégradation UV se sont avérés plus toxiques que la CTC, son espèce auto-phytoplanctonique *Microcystis aeruginosa* et *Scenedesmus obliquus* (Guo *et al.*, 2012). En outre, la présence de substances inorganiques (bicarbonate, phosphate, nitrate et sulfate, etc.) et de matière organique agit comme un piège à radicaux hydroxyles, ce qui diminue l'efficacité de la dégradation.

Les études ont amélioré l'efficacité du traitement UV en combinant avec H_2O_2, l'ozone ou des catalyseurs pour générer plus de radicaux hydroxyles, ce qui a permis d'augmenter l'efficacité de la dégradation.Dans cette technologie hybride, la vitesse de réaction des composés cibles avec des radicaux hydroxyle est souvent supérieure de plusieurs ordres de grandeur à celle de tout oxydant classique. Les études de Yuan *et al.*, 2011 ont permis de réaliser une photodégradation accrue des tétracyclines en utilisant UV/H_2O_2 comparativement aux UV seuls. Considérant le fait que plus d'accumulation de CTC dans les boues d'épuration, le transfert de masse est le principal facteur limitant pour l'application de l'irradiation ultraviolette.

2.2.5.2 Ozonisation

Le traitement ozonation est généralement appliqué pour l'élimination des composés organiques dans l'eau et le traitement des eaux usées. Peu d'études ont été menées sur la dégradation des TC par ozonation et l'évaluation toxicologique des sous-produits. L'élimination complète du CTC a été observée après 90 min d'ozonation à une production de 1,12 mg O_3/min (Kim *et al*., 2012). D'autres études ont montré que l'efficacité élevée de la dégradation de la CTC par couplage avec d'autres procédés de traitement comme O_3/UV et O_3/UV/TiO_2 à un pH optimal de 7 (Hammad Khan *et al*., 2013). L'ozonation partielle a entraîné une augmentation des sous-produits toxiques dans le cas des CTC (Li *et al.*, 2008, Wang *et al.*, 2012). Cependant, le coût de production élevé de l'ozone, une minéralisation non efficace due à la puissance d'oxydation limitée de l'ozone (E^0 = 2,07 V) et un faible taux de transfert de masse limitent les applications industrielles de l'ozonation.

2.2.5.3 Oxydation de Fenton (FO)

Parmi les POA, le FO a plus de capacité à détruire les polluants organiques réfractaires, y compris les antibiotiques dans les matrices complexes. Des études ont démontré le taux de dégradation accru de TC lorsque le peroxyde d'hydrogène est activé avec du Fe^{2+} homogène (Chen *et al*., 2017). L'augmentation de la dose de Fe^{2+} a augmenté la dégradation du TC et réductiondu carbone organique total. Le processus de Fenton et de photo-Fenton a démontré une dégradation de 60% et 80% de TC respectivement en 90 minutes (Yamal-Turbay *et al*., 2013), alors que l'oxydation Fenton hétérogène a éliminé 94% de dégradation en 60 minutes (Hou *et al*., 2016). Le procédé de photo-Fenton n'est généralement pas adapté aux eaux usées avec une forte concentration de matière organique puisque la turbidité empêche la pénétration des rayons UV. Dans ces matrices, le procédé de Fenton était plus efficace même s'il avait une efficacité d'élimination et une minéralisation plus faibles.

2.2.5.4 Ultrasonication

L'application de la technologie de l'ultrasonication au traitement des eaux usées a été développée dernièrement, ce qui se traduit également par un nombre plus restreint de publications concernant le traitement des TC. La production de radicaux hydroxyle par ultrasonication n'est pas efficace comme l'ozonation et les processus FO, et par conséquent, la combinaison avec d'autres POA tels que FO pourrait avoir l'effet synergique dans la minéralisation efficace des composés toxiques. Dans de nombreuses études, le traitement par ultrasons seul a été utilisé pour extraire les TC de différentes matrices (Jacobsen *et al*., 2004, Zhou *et al*., 2009). Cependant, l'effet de l'ultrasonication du bain à 100 W et 20 kHz a été étudié par (Hou *et al*., 2016) pour dégrader TC par activation hétérogène du système Fe_3O_4/H_2O_2. Leurs études ont rapporté l'activation améliorée du H_2O_2 par ultrasons.

Néanmoins, aucune étude n'a été menée sur la dégradation de la CTC dans les boues d'épuration, ce qui pourrait être potentiellement plus toxique. Par conséquent, des études systématiques sont nécessaires pour explorer la dégradation du CTC accumulé dans les boues par adsorption directe aux solides ou par pont métallique. En effet, la solubilisation ou la désintégration des boues par ultrasonication pour libérer la CTC liée forme libre pour une dégradation encore plus efficace doit être étudiée. Une étude de toxicité supplémentaire devrait être réalisée sur les boues issues du procédé d’ultrasons.

2.2.5.5 Oxydation par le persulfate de sodium

La littérature sur la dégradation de la CTC par les radicaux sulfate, est limitée. Actuellement, des recherches sont menées sur l'application du persulfate (PS, $E^0 = 2,01$ V) pour la dégradation des contaminants à l'échelle du banc, ce qui entraîne le développement rapide de cette technologie. Des radicaux sulfates à potentiel redox élevé (SO_4^{-*}, $E^0 = 2,6$ V) peuvent être générés lors de l'activation de PS. De plus, SO_4^{-}* induit en outre la génération du radical hydroxyle. L'effet de la chaleur par ultrasonication ainsi que l’effet du catalyseur de fer sur l'activation de PS ont été testés pour dégrader les TC. Des études de (Hou *et al*., 2012) ont prouvé qu'une concentration optimale de PS et de catalyseur de fer est nécessaire pour une dégradation efficace de

TC. 89,0% de tétracycline a été dégradée après 90 minutes de réaction, à 100 mg/L de CTC, pH à 3,7, PS à 200 mM, fer 1,0 g/L et ultrasons à 80 W. Ledjeri *et al*., 2016 sous forme de Fe^{3+} pour activer PS et confirmer la faisabilité du système PS en raison de l'augmentation de la biodégradabilité des eaux usées traitées.

Cependant, aucune des recherches effectuées jusqu'à ce jour n'a rapporté une dégradation de la CTC par des radicaux sulfates avec l'objectif précis d'éliminer la CTC. Compte tenu des caractéristiques, de la rentabilité, de la nature respectueuse de l'environnement, de la présence naturelle dans l'environnement et de l'activité élevée, il convient de mettre au point un procédé d'assainissement durable des contaminants en utilisant le fer comme catalyseur. Même l'activation du PS à base de fer impliquerait des modes homogènes (Fe^{2+}, soluble) à hétérogènes (Fe^{0}, insoluble) pour activer le persulfate.

2.2.5.6 Dégradation enzymatique

De nombreuses espèces bactériennes et fongiques ont été utilisées pour une dégradation efficace des contaminants récalcitrants. Cependant, les processus de dégradation enzymatique ont été explorés comme une méthode nouvelle et écologique pour l'élimination des contaminants de WW. Jusqu'à présent, très peu de recherches ont été menées sur la dégradation biocatalytique des antibiotiques utilisant des enzymes. Des études récentes ont permis d'appliquer avec succès des champignons à pourriture blanche et leurs enzymes modificatrices extracellulaires pour la dégradation des antibiotiques et pour retracer les contaminants organiques (Margot *et al*., 2013). L'immobilisation de la laccase à l'aide de membranes a démontré une dégradation de TC à 50-60% (24 h) (de Cazes *et al*., 2014), tandis que l'utilisation de médiateurs tels que la laccase de 1-hydroxybenzotriazole a démontré une élimination complète et un taux de TC et de CTC (15 min) (Suda *et al.*, 2012).

Une combinaison d'oxydation biologique et chimique pourrait augmenter l'efficacité de la dégradation et du taux de biodégradation assistée par enzyme. Ces recherches doivent être effectuées pour comprendre la toxicité et la persistance des sous-produits obtenus par cette méthode.

PARTIE 3. PROBLÉMATIQUE

À partir de la littérature sur le sort des tétracyclines dans la STEU, les problèmes suivants ont été soulevés.

- En 1951, la FDA a approuvé l'utilisation de la CTC comme additif pour l'alimentation animale à des fins de promotion de la croissance et de prévention des maladies. La pratique consistant à ajouter de grandes quantités de CTC aux opérations d'alimentation animale et aux écosystèmes agricoles pendant plus de 60 ans, a entraîné une libération constante de CTC dans l'environnement. Bien que les agriculteurs aient utilisé le CTC ***pendant 60 ans***, les scientifiques ont surveillé la ***résistance bactérienne*** seulement pendant les deux dernières décennies. Par conséquent, ***l'efficacité de la CTC chez l'homme a été sérieusement compromise.***
- Une concentration élevée de CTC non métabolisé dans le fumier animal et dans les excrétions humaines a été signalée en ***raison du faible taux de métabolisme et d'absorption (25-30% de la dose) de la CTC dans le tractus gastro-intestinal. L'enfouissement et l'application de biosolides (déchets municipaux) et de fumier comme engrais sur les terres agricoles mènent à l'accumulation de CTC dans le sol.*** Sur la base de l'utilisation actuelle des antibiotiques, la consommation d'antimicrobiens augmentera de 67% d'ici 2030. L'apport constant de CTC bioactif pourrait avoir un impact négatif sur les organismes aquatiques et terrestres.
- La CTC a une forte tendance à se complexer avec des métaux qui sont présents en abondance en les eaux usées et qui modifient les propriétés chimiques (solubilité et coefficient de partage (log P)) de la CTC. L'accumulation de CTC dans les boues d'épuration, en raison de la faible solubilité des complexes métalliques et de la capacité élevée d'adsorption du CTC en boues ajoute un effet synergique à l'accumulation de CTC dans la boue. ***En se basant sur les données de solubilité du CTC (8 mg/ml), on peut s'attendre à ce que la CTC se retrouve dans l'eau et non dans les boues d'épuration. Cependant, la présence de concentrations élevées d'ions métalliques et la capacité d'adsorption élevée de la CTC affecte***

son partitionnement dans la STEU et la recherche n'a pas encore explorée ce domaine.

- La mobilité du CTC et de ses complexes métalliques peuvent avoir une accessibilité différente à travers les membranes cellulaires des bactéries Gram-positives et Gram-négatives en raison des différences inhérentes dans la composition de la paroi cellulaire. Tous les antibiotiques de la famille des TCs ont le même mode d'action (bactériostatique); d'où la possibilité d'une résistance multi-drogue parmi les TCs. La neutralisation de la charge des TCs après complexation peut faciliter une plus grande accumulation de complexes CTC-métal dans les bactéries que la CTC libre. Par conséquent, les complexes CTC-métal ont ***cette activité antibactérienne accrue de CTC par complexation métallique et une activité antibactérienne étendue peut affecter les bactéries nitrifiantes et dénitrifiantes de la station d'épuration. L'adsorption de la CTC à la boue affecte la biodisponibilité de CTC aux microorganismes dans la boue.***
- La STEU crée une approche appropriée pour que les bactéries interagissent en continu avec les antibiotiques à des concentrations sous-inhibitrices et provoquent une résistance dans les populations bactériennes. En outre, les processus biologiques de la STEU qui sont efficaces dans l'élimination des contaminants organiques avec l'azote biologique sont ciblés par les effets bactériostatiques de la CTC. ***Il faut donc étudier les effets possibles de la CTC sur l'élimination biologique de l'azote et le devenir de la CTC dans la station d'épuration.***
- ***L'effet de la complexation sur la persistance et la dégradation de la CTC*** n'a pas encore été complètement exploré. Les études sur la stabilité du complexe CTC-métal avec différents métaux dans des conditions des eaux usées seront utiles ***pour entrevoir le sort de la CTC dans la STEU.***
- Bien que le retrait de la CTC des effluents des eaux usées soit de 90 à 100% (adsorption), ils ne se dégradent pas et s'accumulent dans les boues. Même les procédés de traitement, tels que ***la photolyse, ont entrainé la formation de plus de sous-produits toxiques que la CTC dans l'effluent final.*** Des recherches plus approfondies ont été effectuées sur la CTC (spiked) cible dans les solutions aqueuses. ***Il est donc difficile d'extrapoler les résultats aux conditions réelles.***

Au fur et à mesure que la CTC entre dans la boue, les méthodes de dégradation rapportées dans les solutions aqueuses ne sont plus pertinentes et ne peuvent pas être généralisées aux vraies conditions des boues.

- L'efficacité des POA dépend principalement du contenu organique des eaux usées. Les boues réduisent la pénétration de la lumière et résistent également au transfert de masse, de sorte que les applications ultraviolettes, Fenton et ozone POA ont une efficacité inférieure pour dégrader les contaminants des boues de manière infaillible. De plus, la minéralisation complète des contaminants des boues par POA autonome est coûteuse et peut conduire à la formation de sous-produits toxiques. Une complexation supplémentaire de la CTC avec des métaux peut affecter de nombreux facteurs, tels que ***l'activité antibactérienne, la dégradation et la stabilité de la CTC. Les ions métalliques coexistant et la matrice organique peuvent interagir avec les antibiotiques dans la station d'épuration et peuvent influencer l'élimination globale de la CTC.***

PARTIE 4. HYPOTHÈSE

La présente recherche repose sur l'hypothèse suivante:

1. Selon la littérature, des concentrations élevées de chlorotétracycline ont été trouvées dans les boues ou le fumier animal, et non dans les eaux usées. Une forte chélation avec les métaux et une forte capacité d'adsorption de la CTC affectent le cloisonnement de la CTC dans la station d'épuration. ***L'estimation de la CTC à tous les points d'échantillonnage de la station d'épuration peut aider à se faire une idée du sort de la CTC dans la station d'épuration et à savoir où elle va réellement, c'est-à-dire dans les eaux usées ou les boues d'épuration.***

2. L'estimation des concentrations de métaux dans les eaux usées et boues d'épuration et la corrélation des concentrations de métaux avec une concentration élevée en CTC peuvent favoriser l'accumulation de CTC dans la matrice respective.

3. Les complexes CTC-métal ont une accessibilité différente au microorganisme car les bactéries Gram-positives et Gram-négatives présentent des différences caractéristiques inhérentes dans la composition de la paroi cellulaire. Les complexes CTC-métal ont des constantes d'équilibre différentes à différents pH; ceci peut être soutenu en ayant des groupes d'ionisation multiples dans CTC et même les métaux ont des potentiels ioniques différents, montrant ainsi un équilibre stoechiométrique varié avec CTC. ***L'estimation des constantes de stabilité du CTC avec des métaux en conditions les eaux usées peut prédire la stabilité des complexes CTC-métal dans les conditions réelles des eaux usées.***

4. Indépendamment de l'élimination efficace des contaminants avec l'azote biologique, les souches microbiennes des processus biologiques interagissent en continu avec les antibiotiques à des concentrations sous-inhibitrices. Par conséquent, ces processus pourraient être ciblés par les effets bactériostatiques de la CTC. ***L'estimation des effets possibles de la CTC sur l'élimination biologique de l'azote et le devenir de la CTC peut être utile pour prédire l'effet des antibiotiques sur l'efficacité de la station d'épuration***

5. Malgré une élimination de 90 à 100% de la CTC des effluents des eaux usées, elle ne se dégrade pas, mais s'accumule plutôt dans les boues. ***Comme l'efficacité des POA autonomes dépend principalement de la teneur en matière organique, le pH et l'POA unique peuvent entraîner la formation de sous-produits toxiques.*** Ainsi, les POA hybrides peuvent avoir un effet synergique en augmentant l'efficacité du procédé de traitement dans la dégradation des contaminants organiques, principalement dans les boues, ce qui est plus complexe que les eaux usées.

6. L'échographie POA génère une turbulence qui désintègre les boues et libère le CTC adsorbée dans le liquide de boue. De plus, l'ultra-sonication résulte en un chauffage local intensif et de fortes pressions (cavitation) qui conduit à la formation de radicaux hydroxyles hautement réactifs**. *La turbulence accrue générée par les ultrasons élimine la résistance au transfert de masse, qui est un facteur limitant majeur pour l'application d'POA autonome, tels que les procédés Ultraviolet, Fenton et Ozone.*** Par conséquent, la combinaison de l'oxydation de Fenton et de l'ultrasonication peut conduire à une dégradation plus élevée des contaminants dus à l'effet synergique d'une dissociation accrue du peroxyde d'hydrogène et de la libération de CTC adsorbé pendant l'échographie.

7. Le persulfate (PS, $S_2O8_2^-$) a un potentiel d'oxydation plus élevé (E^0 = 2,01 V) que le H_2O_2 (E^0 = 1,76 V); il est très soluble dans l'eau, relativement peu coûteux et stable. Ces caractéristiques conduiront à une nouvelle approche de l'oxydation chimique de la CTC. Des radicaux sulfate de potentiel redox élevé (SO_4^{-*}, E^0 = 2,6 V) peuvent être générés lors de l'activation de la PS. A température ambiante, le processus d'oxydation du persulfate est lent; Ce procédé peut être accéléré par un catalyseur de fer. ***Ceci peut améliorer la capacité de dégradation de l'oxydant de persulfate en produisant plus de radicaux libres de sulfate avec des radicaux d'hydroxyde.***

8. Les enzymes ligninolytiques, y compris la laccase, peuvent oxyder des produits pharmaceutiques ayant des composés aromatiques phénoliques et non phénoliques des polluants environnementaux (non spécifiques). Le taux de dégradation et l'efficacité de la CTC sont faibles pour le traitement par la laccase seule. Par conséquent, ***la combinaison du traitement par ultra-sonication avec la dégradation de la CTC par la laccase sera explorée comme une méthode écologique pour la dégradation efficace du CTC à partir des eaux usées.***

PARTIE 5. OBJECTIFS

L'objectif global de ce travail est d'étudier le sort de la CTC dans la STEU et de concevoir des méthodes de traitement POA spécifiques pour sa dégradation. Pour atteindre cet objectif global, une série d'objectifs spécifiques à réaliser au cours du travail expérimental est présentée ci-dessous:

1. Estimation de la CTC à différents stades de traitement de la station d'épuration.

1.1 Développement d'une méthode ultrarapide pour la détermination de la concentration de chlorotétracycline dans les eaux usées et les boues d'épuration par LDTD-MS / MS.

1.2 Estimation de la CTC dans les échantillons des eaux usées prélevées dans la station urbaine communautaire de Québec et détermination des concentrations de métaux par spectrométrie de masse à plasma à couplage inductif (ICP-MS). La corrélation entre la concentration élevée en métal et la concentration élevée en CTC dans les échantillons des eaux usées et des boues d'épuration de STEU sera effectuée.

2. Toxicité de la CTC et de ses complexes métalliques

2.1 Les constantes de stabilité de la CTC et de ses complexes métalliques seront déterminées à des conditions de pH des eaux usées par titrage potentiométrique.

2.2 La toxicité de la CTC et de ses complexes métalliques sera évaluée sur des bactéries Gram-positives et Gram-négatives modèles. L'effet des concentrations solides sera déterminé sur la biodisponibilité de CTC en vraie les boues d'épuration.

2.3 L'effet de la chlorotétracycline sur la nitrification et le processus de dénitrification des eaux usées sera également étudié.

3. POA hybrides pour la dégradation de la CTC

3.1 Dégradation de la chlorotétracycline dans la les boues d'épuration en utilisant l'ultra-sonication POA hybride et l'oxydation de Fenton et l'optimisation des paramètres.

3.2 Activation du persulfate par un catalyseur de fer homogène et hétérogène pour une dégradation accrue de la CTC.

3.3 Étude de la cinétique de dégradation de la CTC en combinant le traitement par ultrasons avec la laccase en des eaux usées.

4. Évaluation de la toxicité: La toxicité de la solution résultante par les POA hybrides sera mesurée par le test de levure à l'œstrogène (YES test).

5. Analyse préliminaire des coûts des POAs étudiés: L'évaluation préliminaire des coûts des différents POA hybrides utilisés dans cette étude en fonction des conditions d'exploitation, des exigences et des contraintes sera effectuée.

PARTIE 6. ORIGINALITÉ

A partir des hypothèses et des objectifs ci-dessus, cette étude fait preuve d'originalité en raison des points suivants:

1. Analyse de la CTC en utilisant une méthode analytique nouvelle, rapide et rentable de LDTD-MS/MS pour réduire le temps d'analyse de quelques minutes à quelques secondes.

2. L'analyse des concentrations des métaux dans les eaux usées et les boues d'épuration et la corrélation de l'accumulation de CTC dans les échantillons respectifs

constitueront une nouvelle perspective pour décider du sort du CTC dans la station d'épuration.

3. Détermination des constantes d'équilibre des complexes métalliques CTC aux conditions de pH des eaux usées pour expliquer l'état chimique du CTC dans la station d'épuration.

4. Les différences caractéristiques inhérentes dans la composition de la paroi cellulaire des bactéries Gram-positives et Gram-négatives affectent le transport de la CTC et de ses complexes métalliques à travers la membrane qui détermineront sa toxicité différentielle.

5. Les études de toxicité de la CTC stimulées en vrai les boues d'épuration donneront une idée de la biodisponibilité de CTC aux microorganismes.

6. Les études de l'effet de la CTC sur les processus de nitrification et de dénitrification de la station d'épuration par CTC dans les eaux usées synthétiques expliquent les effets antibiotiques du CTC pendant le traitement biologique.

7. La combinaison d'ultra-sonication et d'oxydation de Fenton POA aura un effet synergique sur la dégradation de la CTC dans les boues d'épuration.

8. Persulfate ($S_2O_8^{2-}$) pour produire les radicaux sulfate de potentiel d'oxydation les plus élevés en tant qu'oxydant chimique dans la dégradation de CTC sera une nouvelle approche. L'accélération du processus d'oxydation du persulfate par un catalyseur de fer homogène et hétérogène peut améliorer la dégradation du CTC.

9. Une combinaison d'ultrasons et de biodégradation assistée par des enzymes de CTC les eaux usées sera un POA hybride rentable, écologique et réalisable qui peut être appliqué dans des stations d'épuration existantes.

L'originalité de la recherche proposée est: ***"*Le devenir et la dégradation de la chlorotétracycline dans les eaux usées et les boues des eaux usées à l'aide de procédés hybrides avancés de bio/oxydation*"*.**

PARTIE 7. SOMMAIRE DES DIFFÉRENTS VOLETS DE RECHERCHE EFFECTUÉS DANS CETTE ÉTUDE

7.1 DÉVELOPPEMENT D'UNE MÉTHODE DE DÉTERMINATION DU PARTITIONNEMENT DE LA CTC DANS LA STEU

7.1.1 Développement d'une méthode rapide et sélective pour la quantification de la CTC dans les échantillons des eaux usées et des boues d'épuration en utilisant la désorption thermique par laser diode couplée à la spectrométrie de masse en tandem (LDTD-MS/MS)

Une quantification précise et rapide de la CTC dans les eaux usées et les boues d'épuration a été effectuée en utilisant la source d'ionisation chimique à pression atmosphérique (APCI) LDTD. Un pic de forte intensité de CTC a été trouvé lorsque le scanner MS/MS a été opéré en mode MRM positif. L'addition d'EDTA peut être effectuée pour une désorption complète de la CTC à partir de la plaque LazWell. La performance de la méthode développée a été validée par l'estimation de la récupération d'extraction, la linéarité, la précision et les limites de détection. Les limites de détection de la méthode étaient de 5,13 µg/L dans les eaux usées et de 7,49 µg/kg dans les boues d'épuration. Les précisions intra et interdiaires étaient respectivement de 4,2% et 7,5% dans les eaux usées et de 9% et 12% dans les boues d'épuration.

7.1.2 ESTIMATION DE LA CONCENTRATION DE LA CTC ET CORRÉLATION DU CLOISONNEMENT DE LA CTC AVEC LA CONCENTRATION DES MÉTAUX DANS LA STEU DE LA STATION DE TRAITEMENT DES EAUX USÉES DE LA COMMUNAUTÉ URBAINE DU QUÉBOUES D'ÉPURATION

La CTC dans la station d'épuration du Québoues d'épuration c a été mesurée en utilisant la méthode LC-MS/MS développée durant cette étude. Une concentration plus élevée de CTC a été mesurée dans les boues d'épuration allant de 430 à 804 µg/kg comparé aux eaux usées allant de 7 à 62 µl/L. La répartition et l'accumulation de CTC dans les boues des eaux usées étaient bien corrélées avec une concentration plus élevée de solides totaux (17,76 g/L) et des concentrations élevées en métaux (Al-354,54 mg/L, Ca-710,29 mg/L, Fe-469,07 mg/L, Mg-86,60 mg/L, Zn-14,04 mg/L) dans les boues par rapport aux eaux usées. Ces résultats ont mis en évidence l'importance des propriétés d'adsorption et de chélation sur le partitionnement de la CTC et posent des questions sur la réutilisation des boues pour l'agriculture et les applications d'enfouissement.

7.2 TOXICITÉ DE LA CTC ET DE SES COMPLEXES MÉTALLIQUES

7.2.1 DETERMINATION DES CONSTANTES DE STABILITE DES COMPLEXES CTC-METAL ET DE LEUR TOXICITE ENVERS LES MICROORGANISMES

La CTC a la capacité de former des complexes métalliques stables ayant pour résultat des propriétés modifiées, telles qu'une faible solubilité, une adsorption accrue et une mobilité plus élevée. Par conséquent, les rapports stoechiométriques des complexes CTC-métal ont été calculés dans des conditions de pH d'eau usée (6,0 ± 0,5). Les constantes de complexation du CTC avec le Ca(II), le Mg(II), le Cu(II) et le Cr(III) ont été étudiées et diminuées dans l'ordre: Mg-CTC> Ca-CTC> Cu-CTC> Cr-CTC. On a trouvé que le rapport molaire pour CTC et le Mg(II) ainsi que le Cu(II) était 1: 1. Ce rapport était de 1: 0,6 pour le Cr(III), et il était de 1: 2 pour le Ca(II). Ces rapports d'équilibre ont été utilisés pour d'autres études de toxicité de la CTC dans des microorganismes.

Selon les résultats de la toxicité, la toxicité aiguë des complexes CTC-métal a diminué dans l'ordre suivant: Mg-CTC> Cu-CTC> Ca-CTC ≡Cr-CTC pour *Bacillus thuringiensis* (Bt), une bactérie Gram positive, et Mg-CTC ≡ Ca-CTC ≡ Cu-CTC ≡ Cr-CTC pour *Enterobacter aerogenes* (Ea), une bactérie Gram négative. Cette différence dans la toxicité est principalement due à la différence dans la composition de la paroi cellulaire et le mécanisme d'absorption des bactéries Gram positives et négatives ainsi que le rôle biologique important des ions métalliques.

7.2.2 L'EFFET DES CONCENTRATIONS DES SOLIDES DANS LES BOUES D'EPURATION SUR LA BIODISPONIBILITE DE CTC

La croissance de Bt et Ea dans les boues secondaires (SS1, 0,99 g/L) et les boues secondaires stabilisées (SS2, 7,13 g/L) avec des solides totaux différents a été diminuée en raison du changement du rapport C et N et de la plus faible disponibilité de carbone soluble et d'azote à partir de la matrice complexe. Les solides en suspension inférieurs (SS1) ont montré une toxicité plus élevée que les solides en suspension (SS2) pour Bt et Ea. Une plus grande capacité d'adsorption de la CTC aux solides de boues a abaissé la toxicité. Par conséquent, l'accessibilité biologique de la CTC aux microorganismes était un facteur clé pour déterminer son activité antibiotique (toxicité).

7.2.3 EFFET DE LA CTC SUR LE PROCESSUS DE NITRIFICATION ET DE DENITRIFICATION DES EAUX USEES

Des expériences de nitrification et de dénitrification ont été réalisées dans les eaux usées biologiques synthétiques et exposées à 0,5 à 100 mg/L de CTC pendant 24 h pour évaluer l'impact aigu sur les activités microbiennes (élimination de l'azote et du carbone organique). Les deux procédés ont été inhibés à des concentrations plus élevées de CTC. Dans la nitrification, l'élimination de la DCO et la cinétique d'oxydation de l'ammoniac ont été inhibées jusqu'à 50% à 10 mg de CTC/L. De même, la cinétique d'oxydation des nitrites a été inhibée à 50% à 0,5 mg de CTC/L. Pendant la dénitrification, 14 et 10 mg/L de CTC ont inhibé l'élimination du DCO et la cinétique d'oxydation des nitrites, respectivement.

7.3 POA HYBRIDES POUR LA DÉGRADATION DE LA CTC

7.3.1 DEGRADATION DE LA CTC DANS LES BOUES D’EPURATION EN UTILISANT L'ULTRA-SONICATION HYBRIDE ET L'OXYDATION DE FENTON POAS ET L'OPTIMISATION DES PARAMETRES

Les procédés d'oxydation de Fenton (FO) et de Ferro-sonication (FS) ont été comparés pour évaluer l'efficacité de la dégradation de la CTC. L'augmentation de la concentration en fer n'a pas montré d'effet significatif ($p > 0,05$) sur la dégradation de la CTC dans les boues, alors qu’une augmentation de la dégradation de la CTC a été observée dans la méthode de FO. L’ajustement du pH des boues à 3 a été suffisant pour générer des radicaux hydroxyles in situ car les boues d’épuration contient suffisamment de concentration en fer (115 mg/kg). L'ultra-sonication (UlS) et le rapport 1: 1 du procédé FO ont montré 67% et 76% de dégradation de la CTC, respectivement, alors que la méthode avec FS a montré 82% de dégradation de la CTC. La libération de CTC par ultrasonication et l’utilisation de fer à pH 3 (FO) ont été les principaux facteurs montrant un effet synergique dans la dégradation de la CTC dans le traitement FS.

7.3.2 ACTIVATION DU PERSULFATE PAR UN CATALYSEUR DE FER HOMOGENE ET HÉTÉROGENE POUR UNE DÉGRADATION SUPÉRIEURE DE LA CTC

L'oxydation de la CTC a été réalisée en utilisant du persulfate (PS, $S_2O8_2^-$) avec Fe^{2+} et du fer zéro-valent (ZVI, Fe^0) comme catalyseurs homogènes et hétérogènes. Les procédés PS/Fe^{2+} et PS/ZVI ont montré une élimination de la CTC de 76% et 94% à un rapport molaire 1: 2 pour 1 µM de CTC en 2 heures à 500 µM de PS et 1000 µM de fer. Plutôt que de générer une production plus élevée de radicaux sulfate à la fois (PS/$Fe^{2\ +}$) avec un catalyseur homogène, la production lente dans le procédé hétérogène (PS/ZVI) est plus efficace.

7.3.3 CINETIQUE DE DEGRADATION DE LA CTC EN COMBINANT LE TRAITEMENT PAR ULTRA-SONICATION AVEC LA LACCASE DANS LES EAUX USEES

La dégradation de la CTC a été réalisée avec des ultrasons assistés par la laccase (UAL). Plus de 80% de la CTC a été éliminé en 2h lorsque le traitement UAL a été utilisé, tandis que la laccase seule a mis 2 jours pour dégrader 87% de la CTC. Le taux de dégradation a augmenté de 14 fois lorsque l'ultra-sonication a été combinée avec la dégradation de la laccase. Par rapport au traitement utilisant la laccase seule, la technique UAL a non seulement augmenté la dégradation de la CTC (~ 80%) mais également réduit le temps de dégradation (2h).

7.4 ÉVALUATION DE LA TOXICITÉ DES ÉCHANTILLONS OBTENUS APRÈS TRAITEMENT PAR POA HYBRIDE PAR DOSAGE À LEVURE DE L'ŒSTROGÈNE (YES)

L'activité des œstrogènes avant et après les échantillons traités par POAs a été réalisée par le test YES. Les échantillons traités par UIS ont montré une activité oestrogénique approximativement égale à 0,35 µg/L de 17ß-oestradiol et les échantillons de FO ont montré une activité œstrogène 17ß-estradiol de 0,1 µg/L. Cette activité oestrogénique élevée des échantillons de boues UIS a expliqué que le traitement UIS semble être plus efficace dans la solubilisation et la libération du composé toxique des solides de boue à phase liquide au lieu de leur dégradation. Les échantillons traités au SF ne présentaient aucune activité œstrogénique. UAL et PS La dégradation de CTC par les POAs avec les UAL et le PS n'a pas montré d'activité oestrogénique avant et après le traitement.

7.5 ÉVALUATION DES COÛTS DES DIFFÉRENTS POA HYBRIDES UTILISÉS DANS CETTE ÉTUDE EN FONCTION DES CONDITIONS D'EXPLOITATION, DES EXIGENCES ET DES CONTRAINTES

En comparant l'efficacité des traitements et la plupart des paramètres de coût, la ferro-sonication hybride était le meilleur processus pour la dégradation des boues des eaux usées ainsi que la dégradation des laccases par ultrasons assistés était le meilleur processus pour le traitement des eaux usées dans les stations de traitement des eaux usées. Dans le cas du traitement des boues des eaux usées, pour un faible coût et une efficacité supérieure (82%), la ferro-sonication a été prise comme la meilleure et pour une faible efficacité (80%) et un faible coût, la dégradation des laccases par ultrasons assistés a été prise en considération pour les eaux usées. Cependant, les technologies d'oxydation avancées sélectionnées et leurs coûts semblent montrer la faisabilité économique dans l'application dans les stations de traitement des eaux usées.

8. REFERENCES

Agwuh KN & MacGowan A (2006) Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. *Journal of Antimicrobial Chemotherapy* 58(2):256-265.

Alaboudi A, Basha EA & Musallam I (2013) Chlortetracycline and sulfanilamide residues in table eggs: Prevalence, distribution between yolk and white and effect of refrigeration and heat treatment. *Food Control* 33(1):281-286.

Ames BD, Lee M-Y, Moody C, Zhang W, Tang Y & Tsai S-C (2011) Structural and biochemical characterization of ZhuI aromatase/cyclase from the R1128 polyketide pathway. *Biochemistry* 50(39):8392-8406.

Arikan OA (2008) Degradation and metabolization of chlortetracycline during the anaerobic digestion of manure from medicated calves. *Journal of Hazardous Materials* 158(2–3):485-490.

Arikan OA, Mulbry W & Rice C (2009) Management of antibiotic residues from agricultural sources: use of composting to reduce chlortetracycline residues in beef manure from treated animals. *Journal of Hazardous Materials* 164(2):483-489.

Auerbach EA, Seyfried EE & McMahon KD (2007) Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water research* 41(5):1143-1151.

Ben W, Qiang Z, Adams C, Zhang H & Chen L (2008) Simultaneous determination of sulfonamides, tetracyclines and tiamulin in swine wastewater by solid-phase extraction and liquid chromatography–mass spectrometry. *Journal of Chromatography A* 1202(2):173-180.

Campagnolo ER, Johnson KR, Karpati A, Rubin CS, Kolpin DW, Meyer MT, Esteban JE, Currier RW, Smith K, Thu KM & McGeehin M (2002) Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. *Science of The Total Environment* 299(1–3):89-95.

Castanon J (2007) History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry science* 86(11):2466-2471.

Chang X, Meyer MT, Liu X, Zhao Q, Chen H, Chen J-a, Qiu Z, Yang L, Cao J & Shu W (2010) Determination of antibiotics in sewage from hospitals, nursery and slaughter house, wastewater treatment plant and source water in Chongqing region of Three Gorge Reservoir in China. *Environmental Pollution* 158(5):1444-1450.

Chen A, Chen Y, Ding C, Liang H & Yang B (2015) Effects of tetracycline on simultaneous biological wastewater nitrogen and phosphorus removal. *RSC Advances* 5(73):59326-59334.

Chen Y-Y, Ma Y-L, Yang J, Wang L-Q, Lv J-M & Ren C-J (2017) Aqueous tetracycline degradation by H2O2 alone: Removal and transformation pathway. *Chemical Engineering Journal* 307:15-23.

Chopra I & Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and molecular biology reviews* 65(2):232-260.

Cirja M, Ivashechkin P, Schäffer A & Corvini PF (2008) Factors affecting the removal of organic micropollutants from wastewater in conventional treatment plants (CTP) and membrane bioreactors (MBR). *Reviews in Environmental Science and Bio/Technology* 7(1):61-78.

Daghrir R, Drogui P, Delegan N & El Khakani MA (2013a) Electrochemical degradation of chlortetracycline using N-doped Ti/TiO2 photoanode under sunlight irradiations. *Water Research* 47(17):6801-6810.

Daghrir R, Drogui P, Delegan N & El Khakani MA (2014) Removal of chlortetracycline from spiked municipal wastewater using a photoelectrocatalytic process operated under sunlight irradiations. *Science of The Total Environment* 466–467:300-305.

Daghrir R, Drogui P & El Khakani MA (2013b) Photoelectrocatalytic oxidation of chlortetracycline using Ti/TiO2 photo-anode with simultaneous H2O2 production. *Electrochimica Acta* 87:18-31.

Daghrir R, Drogui P, Ka I & El Khakani MA (2012) Photoelectrocatalytic degradation of chlortetracycline using Ti/TiO2 nanostructured electrodes deposited by means of a Pulsed Laser Deposition process. *Journal of Hazardous Materials* 199–200:15-24.

de Cazes M, Belleville MP, Petit E, Llorca M, Rodríguez-Mozaz S, de Gunzburg J, Barceló D & Sanchez-Marcano J (2014) Design and optimization of an enzymatic membrane reactor for tetracycline degradation. *Catalysis Today* 236, Part A:146-152.

Directorate VD (2002) Uses of Antimicrobials in Food Animals in Canada: Impact on Resistance and Human Health. *Report of the Advisory Committee on Animal Uses of Antimicrobials and Impact on Resistance and Human Health. HealthCanada*.

Dröge M, Pühler A & Selbitschka W (2000) Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Molecular and General Genetics MGG* 263(3):471-482.

Eisner H & Wulf R (1963) The metabolic fate of chlortetracycline and some comparisons with other tetracyclines. *Journal of Pharmacology and Experimental Therapeutics* 142(1):122-131.

Food U & Administration D (2010) Summary report on antimicrobials sold or distributed for use in food-producing animals. (FDA, Washington, DC).

Gao P, Ding Y, Li H & Xagoraraki I (2012a) Occurrence of pharmaceuticals in a municipal wastewater treatment plant: Mass balance and removal processes. *Chemosphere* 88(1):17-24.

Gao P, Munir M & Xagoraraki I (2012b) Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Science of The Total Environment* 421–422:173-183.

Gavalchin J & Katz SE (1994) The persistence of fecal-borne antibiotics in soil. *Journal of AOAC international* 77(2):481-485.

Gu C & Karthikeyan K (2005) Interaction of tetracycline with aluminum and iron hydrous oxides. *Environmental science & technology* 39(8):2660-2667.

Gu C, Karthikeyan K, Sibley SD & Pedersen JA (2007) Complexation of the antibiotic tetracycline with humic acid. *Chemosphere* 66(8):1494-1501.

Guerra W, Silva IR, Azevedo EA, Monteiro ARdS, Bucciarelli-Rodriguez M, Chartone-Souza E, Silveira JN, Fontes AP & Pereira-Maia EC (2006) Three new complexes of platinum (II) with doxycycline, oxytetracycline and chlortetracycline and their antimicrobial activity. *Journal of the Brazilian Chemical Society* 17(8):1627-1633.

Guo RX & Chen JQ (2012) Phytoplankton toxicity of the antibiotic chlortetracycline and its UV light degradation products. *Chemosphere* 87(11):1254-1259.

Hamad B (2010) The antibiotics market. *Nature Reviews Drug Discovery* 9(9):675-676.

Hammad Khan M, Jung H-S, Lee W & Jung J-Y (2013) Chlortetracycline degradation by photocatalytic ozonation in the aqueous phase: mineralization and the effects on biodegradability. *Environmental technology* 34(4):495-502.

Hirsch R, Ternes T, Haberer K & Kratz K-L (1999) Occurrence of antibiotics in the aquatic environment. *Science of the Total Environment* 225(1):109-118.

Hou L, Wang L, Royer S & Zhang H (2016) Ultrasound-assisted heterogeneous Fenton-like degradation of tetracycline over a magnetite catalyst. *Journal of Hazardous Materials* 302:458-467.

Hou L, Zhang H & Xue X (2012) Ultrasound enhanced heterogeneous activation of peroxydisulfate by magnetite catalyst for the degradation of tetracycline in water. *Separation and Purification Technology* 84:147-152.

It H (Estimates of Antimicrobial Abuse in Livestock-The use of antimicrobials in animal agri-culture is eroding physicians’ ability to treat infectious disease in humans. By Margaret Mellon, Charles Benbrook, and Karen Lutz Benbrook. UCS, 2001.).

Jacobsen AM, Halling-Sørensen B, Ingerslev F & Hansen SH (2004) Simultaneous extraction of tetracycline, macrolide and sulfonamide antibiotics from agricultural soils using pressurised liquid extraction, followed by solid-phase extraction and liquid chromatography–tandem mass spectrometry. *Journal of chromatography A* 1038(1):157-170.

Jia Y, Liu J, Cha S, Choi S, Park YC & Liu C (2017) Magnetically separable Au-TiO2/nanocube ZnFe2O4 composite for chlortetracycline removal in wastewater under visible light. *Journal of Industrial and Engineering Chemistry* 47:303-314.

Jiang L, Hu X, Yin D, Zhang H & Yu Z (2011) Occurrence, distribution and seasonal variation of antibiotics in the Huangpu River, Shanghai, China. *Chemosphere* 82(6):822-828.

Karcı A & Balcıoğlu IA (2009) Investigation of the tetracycline, sulfonamide, and fluoroquinolone antimicrobial compounds in animal manure and agricultural soils in Turkey. *Science of the total environment* 407(16):4652-4664.

Karthikeyan KG & Meyer MT (2006) Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Science of The Total Environment* 361(1–3):196-207.

Kemper N, Färber H, Skutlarek D & Krieter J (2008) Analysis of antibiotic residues in liquid manure and leachate of dairy farms in Northern Germany. *agricultural water management* 95(11):1288-1292.

Khan MH & Jung J-Y (2016) Ozonation of chlortetracycline in the aqueous phase: Degradation intermediates and pathway confirmed by NMR. *Chemosphere* 152:31-38.

Kim I & Tanaka H (2009) Photodegradation characteristics of PPCPs in water with UV treatment. *Environment International* 35(5):793-802.

Kim T-H, Kim SD, Kim HY, Lim SJ, Lee M & Yu S (2012) Degradation and toxicity assessment of sulfamethoxazole and chlortetracycline using electron beam, ozone and UV. *Journal of Hazardous Materials* 227–228:237-242.

Ledjeri A, Yahiaoui I & Aissani-Benissad F (2016) The electro/Fe3+/peroxydisulfate (PDS) process coupled to activated sludge culture for the degradation of tetracycline. *Journal of Environmental Management* 184, Part 2:249-254.

Li K, Yediler A, Yang M, Schulte-Hostede S & Wong MH (2008) Ozonation of oxytetracycline and toxicological assessment of its oxidation by-products. *Chemosphere* 72(3):473-478.

Lindsey ME, Meyer M & Thurman E (2001) Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography/mass spectrometry. *Analytical chemistry* 73(19):4640-4646.

Margot J, Bennati-Granier C, Maillard J, Blánquez P, Barry DA & Holliger C (2013) Bacterial versus fungal laccase: potential for micropollutant degradation. *AMB Express* 3(1):63.

Martínez-Carballo E, González-Barreiro C, Scharf S & Gans O (2007) Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. *Environmental Pollution* 148(2):570-579.

Motoyama M, Nakagawa S, Tanoue R, Sato Y, Nomiyama K & Shinohara R (2011) Residues of pharmaceutical products in recycled organic manure produced from sewage sludge and solid waste from livestock and relationship to their fermentation level. *Chemosphere* 84(4):432-438.

Miller JH, Novak JT, Knocke WR & Pruden A (2016) Survival of antibiotic resistant bacteria and horizontal gene transfer control antibiotic resistance gene content in anaerobic digesters. Frontiers in microbiology 7.

Munir M, Wong K & Xagoraraki I (2011) Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water research* 45(2):681-693.

Organization WH (1988) The world drug situation.

Orth P, Saenger W & Hinrichs W (1999) Tetracycline-Chelated Mg2+ Ion Initiates Helix Unwinding in Tet Repressor Induction†. *Biochemistry* 38(1):191-198.

Puicharla R, Mohapatra D, Brar S, Drogui P, Auger S & Surampalli R (2014) A persistent antibiotic partitioning and co-relation with metals in wastewater treatment plant—chlortetracycline. *Journal of Environmental Chemical Engineering* 2(3):1596-1603.

Pulicharla R, Brar S, Drogui P, Verma M & Surampalli R (2015a) Removal Processes of Antibiotics in Waters and Wastewaters: Crucial Link to Physical-Chemical Properties and Degradation. *Journal of Hazardous, Toxic, and Radioactive Waste* 19(4):04015008.

Pulicharla R, Brar SK, Rouissi T, Auger S, Drogui P, Verma M & Surampalli RY (2017) Degradation of chlortetracycline in wastewater sludge by ultrasonication, Fenton oxidation, and ferro-sonication. *Ultrasonics Sonochemistry* 34:332-342.

Pulicharla R, Das RK, Brar SK, Drogui P, Sarma SJ, Verma M, Surampalli RY & Valero JR (2015b) Toxicity of chlortetracycline and its metal complexes to model microorganisms in wastewater sludge. *Science of the Total Environment* 532:669-675.

Qiang Z & Adams C (2004) Potentiometric determination of acid dissociation constants (pK a) for human and veterinary antibiotics. *Water research* 38(12):2874-2890.

Salazar-Rábago JJ, Sánchez-Polo M, Rivera-Utrilla J, Leyva-Ramos R & Ocampo-Pérez R (2016) Role of 1[O2]⁎ in chlortetracycline degradation by solar radiation assisted by ruthenium metal complexes. *Chemical Engineering Journal* 284:896-904.

Sarmah AK, Meyer MT & Boxall ABA (2006) A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* 65(5):725-759.

Siddappa K, Mane SB & Manikprabhu D (2014) Spectral characterization and 3D molecular modeling studies of metal complexes involving the O, N-donor environment of quinazoline-4 (3H)-one Schiff base and their biological studies. *The Scientific World Journal* 2014.

Suda T, Hata T, Kawai S, Okamura H & Nishida T (2012) Treatment of tetracycline antibiotics by laccase in the presence of 1-hydroxybenzotriazole. *Bioresource Technology* 103(1):498-501.

Tran N, Drogui P, Nguyen L & Brar SK (2016) Electrooxidation–Ultrasonication Hybrid Process for Antibiotic Chlortetracycline Treatment. *Journal of Environmental Engineering* 142(5):04016011.

Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A & Laxminarayan R (2015) Global trends in antimicrobial use in food animals. *Proceedings of the National Academy of Sciences* 112(18):5649-5654.

Wang Y, Zhang H, Chen L, Wang S & Zhang D (2012) Ozonation combined with ultrasound for the degradation of tetracycline in a rectangular air-lift reactor. *Separation and Purification Technology* 84:138-146.

Watkinson AJ, Murby EJ, Kolpin DW & Costanzo SD (2009) The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. *Science of The Total Environment* 407(8):2711-2723.

Wei R, Ge F, Huang S, Chen M & Wang R (2011) Occurrence of veterinary antibiotics in animal wastewater and surface water around farms in Jiangsu Province, China. *Chemosphere* 82(10):1408-1414.

Wouters M & van Leeuwen F (2005) Chlortetracycline and tetracycline. *WHO Food Additive Series* 36.

Yamal-Turbay E, Jaén E, Graells M & Pérez-Moya M (2013) Enhanced photo-Fenton process for tetracycline degradation using efficient hydrogen peroxide dosage. *Journal of Photochemistry and Photobiology A: Chemistry* 267:11-16.

Yang S & Carlson K (2003) Evolution of antibiotic occurrence in a river through pristine, urban and agricultural landscapes. *Water Research* 37(19):4645-4656.

Yuan F, Hu C, Hu X, Wei D, Chen Y & Qu J (2011) Photodegradation and toxicity changes of antibiotics in UV and UV/H 2 O 2 process. *Journal of Hazardous Materials* 185(2):1256-1263.

Yuan X, Qiang Z, Ben W, Zhu B & Liu J (2014) Rapid detection of multiple class pharmaceuticals in both municipal wastewater and sludge with ultra high performance liquid chromatography tandem mass spectrometry. *Journal of Environmental Sciences* 26(9):1949-1959.

Zhang H, Zhang M & Gu G (2008) Residues of tetracyclines in livestock and poultry manures and agricultural soils from North Zhejiang Province. *J Ecol Rural Environ* 24(3):69-73.

Zhao L, Dong YH & Wang H (2010) Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. *Science of The Total Environment* 408(5):1069-1075.

Zhao Y, Geng J, Wang X, Gu X & Gao S (2011) Adsorption of tetracycline onto goethite in the presence of metal cations and humic substances. *Journal of colloid and interface science* 361(1):247-251.

Zhou J, Xue X, Li Y, Zhang J, Chen F, Wu L, Chen L & Zhao J (2009) Multiresidue determination of tetracycline antibiotics in propolis by using HPLC-UV detection with ultrasonic-assisted extraction and two-step solid phase extraction. *Food Chemistry* 115(3):1074-1080.

CHAPTER 2

METHOD DEVELOPMENT AND DETERMINATION OF CTC PARTITIONING IN WWTP

PART 1

REMOVAL PROCESSES OF ANTIBIOTICS IN WATERS AND WASTEWATERS – CRUCIAL LINK TO PHYSICAL-CHEMICAL PROPERTIES AND DEGRADATION

Rama Pulicharla[a], S.K. Brar[a], P. Drogui[a], M. Verma[b], R.Y. Surampalli[c]

[a]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

[b]CO2 Solutions Inc., 2300, rue Jean-Perrin, Québec, Québec G2C 1T9 Canada

[c]Department of Civil Engineering, University of Nebraska-Lincoln, N104 SEC PO Box 886105, Lincoln, NE 68588-6105, US

(*Corresponding author, Telephone: + 418 654 3116; Fax: + 418 654 2600; Email: satinder.brar@ete.inrs.ca)

Journal of Hazardous, Toxic, and Radioactive Waste, (2015)19:(4)

Résumé

La présence de résidus d'antibiotiques dans l'environnement est une problématique de pollution émergente en raison de leur libération ininterrompue et démesurée dans l'environnement et aussi à cause de leurs effets néfastes même à de faibles concentrations. L'apparition et le sort de ces contaminants traces pendant le traitement des eaux usées sont des problèmes clés dans le recyclage des ressources en eau. L'une des principales préoccupations est la présence d'antibiotiques dans l'eau potable suite au traitement des eaux usées. Plusieurs processus ont été adaptés pour dégrader / éliminer les antibiotiques efficacement. Cette revue donne un aperçu sur l'efficacité de divers procédés d'oxydation avancés pour l'élimination des antibiotiques et leur lien crucial avec leurs propriétés physico-chimiques et leur dégradation.

Mots-clés: Antibiotiques; Contaminants; Traitement des eaux usées; Processus d'oxydation avancés; Stratégie combinée.

Abstract

The presence of antibiotic residues in the environment is an emerging pollution issue due to their uninterrupted and unrestrained release into the environment and its harmful effects even at low concentrations. The occurrence and fate of these trace contaminants during wastewater treatment are key issues in the recycle of water resources. One of the major concerns is the presence of antibiotics in drinking water subsequent to wastewater treatment. Several processes have been adopted to degrade/eliminate antibiotics efficiently. This review provides an insight on efficacy of various advanced oxidation processes for the removal of antibiotics and its crucial link to their physical-chemical properties and degradation.

Keywords: Antibiotics; Contaminants; Wastewater treatment; Advanced oxidation processes; Combination strategy.

Introduction

Antibiotic usage has received a lot of awareness over the last several years due to the ever-increasing water borne diseases. And it has become challenging for traditional water treatment technologies to adapt to their removal. Antibiotics enter the environment (soil, surface waters, ground and drinking water) through crudely treated wastewater (WW) discharges or from improperly controlled manufacturing/production facilities (Karthikeyan and Meyer 2006; Larsson et al. 2008) or unmetabolized/active metabolites from humans or animals excretions (Huang et al. 2001; Darlymple et al. 2007; Klavarioti et al. 2009; Kümmerer 2009).

Once residual antibiotics enter the environment, fate of these antibiotics depends upon the nature, physical and chemical properties of the antibiotics. Biodegradable antibiotics get ultimately mineralized to carbon dioxide and water or metabolizes to a more hydrophilic molecule which passes through the wastewater treatment plant ends up in the watershed. However, non-biodegradable antibiotics do not degrade readily and are partially retained in the sedimentation sludge (Klavarioti et al. 2009; Rizzo et al. 2013). Persistence of residual pharmaceuticals in the environment constitutes a serious environmental problem as they may impose serious toxic effects on humans and other living organisms (Martinez 2009). The treatment processes create an approach suitable for bacteria to mix continuously with antibiotics at sub-inhibitory concentrations and cause resistance in bacterial populations (Hirsch et al. 1999; Mira et al. 2003; Kemper 2008).

Recent studies demonstrated that the conventional processes have varying removal rates for antibiotic residues (Batt et al. 2006; Clara et al. 2005; Karthikeyan and Meyer 2006; Le-Minh et al. 2010), ranging from less than 20% to greater than 90% and instead generate a new waste. Further, despite the 90% removal, there is possibility of formation of by-products which can be more toxic than the original antibiotic. In comparison, advanced oxidation processes (AOPs), such as photolysis using ultraviolet (UV) light, hydrogen peroxide combined with UV (UV/H_2O_2), Fenton (H_2O_2/Fe^{2+}) and photo-Fenton treatment ($H_2O_2/Fe^{2+}/UV$), ozonation, semiconductor photocatalysis and electrochemical processes can achieve higher removal rates for antibiotics and also reduce the toxicity of by-products (Reungoat et al. 2012; Babić et al. 2013; Wu et al. 2013). These processes are pH dependent, applicable to low total organic carbon (TOC) effluents and flow rates (Balcioğlu and Ötker 2003; Homem and Santos 2011).

In recent times, research has been directed towards the design of wastewater treatment technologies for efficient removal of residual antibiotics. Applying a combination strategy of physicochemical and biological treatments may improve treatment efficiencies. This may eliminate the highly biodegradable part of the wastewaters and also degrade the recalcitrant contaminants (Klavarioti et al. 2009; Oller et al. 2011). The aim of this review is to evaluate the effectiveness of advanced oxidant technologies for efficient removal of antibiotic residues from wastewater and sewage sludge and its crucial link to their physical-chemical properties and eventually degradation.

Generalities

Today, a broad range of naturally occurring and synthetic antibiotics are used which are designed to be act very effectively even at low doses. This results in increased consumption of antibiotics which enter into the terrestrial and aquatic environment primarily via the discharge of raw and treated sewage from residential users, drug manufacturing plants, medical facilities, excretion (urine and feces) and the topically applied pharmaceuticals (when washed off). In fact metabolized/unmetabolized forms of antibiotics entering the environment, depending on the route of discharge into water sources (Kolpin et al. 2004) also have the ability to bioaccumulate and magnify. Residues of antibiotics in soil resulted from the use of contaminated excrements as fertilizer on agriculture land. Surface run off and leaching of antibiotics from soils act as a source of antibiotic contaminants for the aqueous environment (Ostermann et al. 2013). Apart from therapeutic use; some of these antibiotics are used as growth promoters, this leads to massive usage of antibiotics (Fuentefria et al. 2011) which possibly have noticeable impact on the environment (Kümmerer 2004; Vignesh et al. 2012; Ocsana et al. 2013). Figure 2.1.1 gives a picture of antibiotics as third largest group among all pharmaceuticals in the world market 2012 in U.S. (united states).

Classification of antibiotics

Antibiotics are generally classified according to their chemical structure or mode of action. Antibiotics sharing similar chemical structure will generally show similar patterns of antibacterial activity, effectiveness, side effects and fate in wastewater treatment. Table 2.1.1 classifies antibiotics based on their chemical structure and mode of action. Log P (partition coefficient) is one of the important chemical properties which affect the partitioning of antibiotics between WW and WWS in a WWTP.

Occurrence and fate of antibiotics in wastewater

Antibiotics are used extensively as medicine and also in aquaculture, for the purpose of preventing (prophylaxis) or treating microbial infections (Kemper 2008). Once the antibiotics enter the environment, they transform into different forms through various processes, such as sorption, abiotic and biotic processes. These processes directly influence the fate of these compounds in the environment as well as their biological activities. The pictorial representation of principal routes of contamination and recycling of antibiotics is shown in Figure 2.1.2.

A significant amount of antibiotics pass in the environment through excretion (Alcock et al. 1999; Hirsch et al. 1999; Aksu and Tunc 2005); subsequently, the concentration may rise up to ppm levels. Pharmaceutically active forms of antibiotics may promote the development of bacterial resistance. The fate of each antibiotic in the environment remains a unique situation that requires investigation. Table 2.1.2 describes the behavior of antibiotics in different water matrices for a given treatment method. As summed in the table 2.1.2, the degradation efficiency of treatment method is mainly dependent on the wastewater matrix composition (concentration and type of ions, organic matter). The formation of by-products and their toxicity in the WW are principally determined by the chemical properties of antibiotics and the treatment method (as discussed later in table 2.1.5). Therefore, the concentration of antibiotics, transformation products and/or pathways should be included in the future studies in order to obtain accurate information on the removal technologies (Michael et al. 2012).

Moreover, the presence of antibiotics in natural systems even in low concentrations leads to the development of multi-resistant strains of bacteria (Davison 1999; Vaz-

Moreira et al. 2012; Novo et al. 2013). Hence, it is necessary to treat the effluents containing antibiotics effectively before discharging into water sources. Apart from the water and WW matrices (as discussed in the table 2.1.2), the physico-chemical properties and concentration of antibiotics in WW also play a crucial role in the degradation (explained in the table 2.1.3). Factors affecting the antibiotic degradation are presented in table 2.1.3.The degradation efficiency of the same method varies for diverse antibiotics.

Worldwide regulations of antibiotics in the environment

Antibiotics have always been considered one of the phenomenal discoveries of the 20th century. The successful use of any antibiotic is not to develop tolerance or resistance from the time it is first employed. At present, we are totally dependent on antibiotics for the treatment of any infectious diseases. There is a growing international threat about the potential impact of antibiotic residues on the environment. The lack of knowledge on the genetic capacities of microbes and the massive usage of antibiotics leads to the development of antibiotic resistance. (Davies 1996). Most of the national and international organizations conducted meetings, workshops and working groups for gathering information on worldwide antibiotic resistance. Many resolutions and recommendations have been put forward, but often with a limited impact due to a lack of coordination among them. Only a few developed countries worldwide have managed to reduce antibiotic consumption, albeit at a small scale.

Continuous disposal of antibiotics into the environment over the past few years places the world in real danger. Hence, it is important to develop strategies for addressing this issue and to follow guidelines and regulations to restrict the use of antibiotics in future to protect humans and livestock. In 1951, Food and Drug Administration (FDA) had allowed the use of antibiotics for veterinary purpose. But, by 1970s, due to public health issues and development of antibiotic resistant infections caused by the indiscriminate use of antibiotics in humans and animals, it led to a global threat. Later, in 1977, FDA announced that it would begin banning usage of some agricultural and livestock antibiotics. However, this was never implemented as it was proved that antibiotics were necessary to keep animals in good health. The U.S. FDA is taking its biggest step to support the judicious use of antimicrobial drugs in food-producing animals. In collaboration with other government agencies, FDA developed a framework to “preserve the effectiveness of medically important antimicrobials for treating diseases in humans” and limited use of important antimicrobial drugs in food-producing animals. The National Antimicrobial Resistance Monitoring System (NARMS) program was established in 1996 as a partnership between the U.S. FDA, the Centers for Disease Control and Prevention (CDC), and the U.S. Department of Agriculture (USDA). The programme monitors the trends and research in antibiotic resistance and assists the FDA in making decisions related to the approval of safe and effective antimicrobial drugs for animals. In 2005, FDA prohibited the use of fluoroquinolones in animal husbandry due to high levels in poultry further leading to drug resistance in humans. New York ordered the FDA in 2012 to begin the process to ban the indiscriminate agricultural usage of penicillin and tetracycline because of dangers to human health and also announced restrictions on agricultural uses of cephalosporin (Cefzil and Keflex).

McDonald’s Global Policy on Antibiotic use in food animals proposed guiding principles for sustainable uses of antibiotics (McDonald’s Global Antibiotics Policy, 2003). And personnel who supervise use in animals and humans must work to sustain the long-term efficacy of antibiotics. This policy is under active review by scientists and regulators around the world. McDonalds banned the use of antibiotic belonging to the classes of compounds approved for use in human medicine when used solely for growth promotion purposes. Continuous low-dose administration of antibiotic agents as growth promoters (increase the rate and efficiency of weight gain) in healthy livestock (mostly in the production of pigs, broiler chickens, turkeys, and feedlot cattle) favors the spread of resistant bacteria. Due to poor absorption of antibiotics in the gut of the animals, the majority is excreted unchanged in feces and urine. And further land application of these animal wastes as fertilizer is a growing international concern about the potential impact of antibiotic residues on the environment (Sarmah et al. 2006). The first call for a ban on non-therapeutic use of antibiotics in animals and agriculture was proposed by Swann committee in 1969, UK. Figure 2.1.3 presents the consumption patterns of antibiotics in humans and livestock. In three years, from 2009 to 2011, there is a decrease in usage of antibiotics in humans mainly due to increase in resistance because of massive usage of them in livestock.

Antibiotic resistance in Canada was first addressed by Health Canada Consensus Conference held in Montreal in 1997. The aim of this conference was establishment of a national surveillance system to monitor antimicrobial resistance. This proposal led to establishment of Integrated Action Plan for Canadians on Controlling Antimicrobial Resistance (CCAR) and also an "Advisory Committee on animal uses of antimicrobials in Canada: Impact on resistance and human health", which reported in 2002 and 2004 respectively. The United States is Canada's major trading partner in food animal products. It is important that regulatory standards be considered from an equivalence standpoint. Main activities of CCAR's include resistance surveillance, optimal antibiotic use, and infection prevention and control. CCAR reported 14% decrease in the overall number of antibiotic prescriptions dispensed between 1995 and 2000, indicating the need for continued efforts to promote appropriate prescriptions. Canadian Veterinary Medical Association (CVMA) encourages veterinarians to follow the guidelines on the prudent or judicious use of antimicrobials and provides advice on the alternatives to antibiotics. Also, in collaboration with CCAR, the CVMA is now looking at developing species-specific guidelines on the prudent use of antimicrobial drugs. The Canadian Animal Health Institute (CAHI) is a trade association of animal drug makers was formed in 1999. This group is gathering information relevant to formulate meaningful recommendations related to nonhuman use of antimicrobials (Conly 2001).

On January 1st 2006, the EU banned the feeding of all antibiotics and related drugs to livestock for growth promotion purposes. The European Food Safety Authority (EFSA) proposed maximum residues levels (MRLs), these new rules would require that companies demonstrate the additive's positive effect for the animals (efficacy) and the absence of risk for human health, animal health and the environment (safety). The Belgian Antibiotic Policy Coordination Committee (BAPCOC) organized several national campaigns and resulted in 36% decrease in antibiotic prescription between 1999 and 2007 (Goossens et al. 2008).

In actuality, the world without antibiotics is not ready as yet. Scientists are trying to found alternatives for antibiotics. Modifications in the regulatory procedures and stringent guidelines should be followed to control the usage of antibiotics. FAO/OIE/WHO has jointly developed international scientific regulations and standards for addressing antibiotic resistance worldwide.

Treatment technologies for removal of antibiotics from wastewater

Advanced oxidation processes (AOPs)

Wastewater effluents containing antibiotic residues are mostly unregulated, recalcitrant compounds and are difficult to detoxify and biodegrade by conventional and traditional biological treatments. Applying AOPs for these types of compounds is an efficient alternative. AOPs are well-known technologies for the treatment of pharmaceuticals in WW, where the pollutants are chemically oxidized by hydroxyl free radicals (Elmolla and Chaudhuri 2009a,b; Lin et al. 2009; Elmolla et al. 2010; Renato et al. 2010; Jin et al. 2012). The principle of AOPs (including O_3/H_2O_2, UV/O_3, UV/H_2O_2, H_2O_2/Fe^{2+}, and UV-TiO_2) is to produce hydroxyl radical in water, a very powerful oxidant capable of oxidizing a wide range of organic compounds with one or more double bonds, such as antibiotics (Khan 2010; Hapeshi et al. 2013; Rodrigues-Silva et al. 2013). Any standalone AOP may not be totally efficient for complete mineralization of organic contaminants and also it may lead to the formation of more toxic byproducts (Gómez-Ramos Mdel et al. 2011; Prados-Joya et al. 2011). Studies showed that photolysis was mainly limited by the time course of TOC and chemical composition of wastewater on which pH depends (Jiao et al. 2008; Avisar et al. 2010; Prados-Joya et al. 2011) and also higher antibiotic concentration. Performance of lone oxidation processes depended on many parameters, hence it has limited efficiency. In this context, hybrid technologies gain more advantage as discussed in subsequent sections.

UV/Photolysis AOP

UV/photolysis (direct photolysis) is an AOP process in which target compounds absorb photons and this oxidation process induced by light. Effectiveness of an irradiation can be improved by combining with H_2O_2, ozone or catalysts; whose photolytic degradation generates more hydroxyl radicals thus facilitating the degradation (also referred as indirect photolysis). This is an attractive technology because the reaction rate of target compounds with hydroxyl radicals is often several orders of magnitude higher than with any conventional oxidant.

Yuan et al. (2011) concluded that increased photodegradation of tetracyclines and TOC were achieved by using UV/H_2O_2 compared to UV alone and also evaluated the toxicity of photolytic byproducts of light sensitive tetracycline group antibiotics. This study showed the critical role of initial antibiotic concentration, pH and TOC and also higher risk of the degraded products. By using UV/H_2O_2, 98–99% of tetracycline antibiotics (oxytetracycline (OTC), doxycycline (DTC)) and 11% to 15 % of TOC were removed where with UV irradiation 3 – 4 % of TOC was removed. The photodegradation rate of a compound depends on target compound's light absorption rate and quantum yield, UV lamp technology (intensity and frequency), H_2O_2 and O_3 concentration (if used) and the type of matrix. The latter considered action being very important; presence of inorganic (bicarbonate, phosphate, nitrate and sulfate, etc.) and organic matter (García-Galán et al. 2012) acts as a radical scavenger, thus decreasing degradation.

Ozone AOP

Ozone is classified as an AOP; ozone decomposes in water to form hydroxyl radicals which are stronger oxidizing agents than ozone itself. The target compounds are oxidized by the direct ozone and hydroxyl radicals. The target compound reaction directly with ozone is selective electrophilic mechanism whereas the reaction with hydroxyl radicals is non-selective and the reaction rate is higher than ozone molecule. Initial dissociation of ozone in water for hydroxide radical generation is as follows:

$O_3 + OH^- \;\text{---->}\; O_2 + HO_2^-$ -- (1)

$O_3 + HO_2^- \;\text{---->}\; HO_2^{\bullet} + O_3^{\bullet}$ -- (2)

According to the reactions (1) and (2), the dissociation of ozone is mainly pH dependent and this can be artificially accelerated by increasing the pH of WW. Moreover, treatment performance is enhanced if ozone is combined with UV, H_2O_2 (Balcioğlu and Ötker 2003; Lin et al. 2009) or with catalysts (Fernando et al. 2008) to increase the production of hydroxyl radicals. Coupling ozonation with H_2O_2 (perozonation) is more efficient in turbid WW effluents, compared to photolysis of ozone process as penetration of light is restricted (Serpone et al. 2010). As mentioned in the table 2.1.4, combination of ozonation with H_2O_2 achieved 100% COD removal (Balcioğlu and Ötker 2003). However, optimization of H_2O_2 concentrations is necessary, since it may act as a free radical scavenger at higher concentration. On comparison with other oxidative methods, it requires a larger amount of oxidant, is extremely pH-dependent and high cost of equipment and energy is required. Apart from this, the toxicity of effluents remains as such or even increases. For these reasons, ozonation must be combined with appropriate techniques for the best economic and ecological performance.

Ozonation and activated carbon (AC) combined processes increases antibiotic removal rate and the volume of WW treated, reduces the concentration of dissolved organic matter (DOM) (Reungoat et al. 2012) and the quantity of AC required.

UV /H_2O_2 / O_3 Combined technologies

One way to increase the oxidation process efficiency is conjugation among AOPs even though these methods have been proved to have their own limitations and are not 100% efficient. Various combinations of UV, O_3 and H_2O_2 have been tested for complete removal of antibiotics from WW effluents (Lee et al. 2011; Lester et al. 2011). Among these studies, Lester et al. (2011) reported, addition of H_2O_2 to UV/O_3 showed almost similar degradation of ciprofloxacin (CIP) with time but in the case of cyclophosphamide (CPD), degradation increased with the addition of H_2O_2 to UV/O_3. Hence, the degradation efficacy of each AOP was compound dependent. Lee et al. (2011) investigated the performance of different combinations of oxidation agents and showed the order of removal rate as: $O_3 < O_3/H_2O_2 < O_3/UV < O_3/UV/H_2O_2$ for chlorotetracycline from livestock effluents. These studies also explained the importance of H_2O_2 concentration for the UV/H_2O_2 and H_2O_2/O_3 process where H_2O_2 acted as an $OH^•$ initiator at lower concentrations and as an $OH^•$ scavenger at higher concentrations.

Fenton and photo-Fenton oxidation

In 1894, Henry John Horstman Fenton discovered special oxygen transfer properties of several metals which have strong catalytic power to generate highly reactive hydroxyl radicals via a free radical chain reaction. Since then, the iron catalyzed hydrogen peroxide has been called Fenton's reaction. Iron-salt-dependent decomposition of hydrogen peroxide, generating the highly reactive hydroxyl radicals is as follows:

$Fe^{2+} + H_2O_2 \text{---->} Fe^{3+} + OH^{\bullet} + OH^{-}$ --- (1)

$Fe^{3+} + H_2O_2 \text{---->} Fe^{2+} + OOH^{\bullet} + H^{+}$ --- (2)

Generation of hydroxyl radicals explains that the process efficiency is closely related to the solution pH. In fact many authors studied the effect of wide range of pH on the fate of regeneration of active iron (Fe^{2+}), and hydrogen peroxide. The optimum values for this AOP process was between 2 – 4. At low pH, hydrogen peroxide stability increased due to the formation of oxonium ions ($H_3O_2^{+}$) which led to its reduction in reactivity with ferrous ions (Elmolla and Chaudhuri 2009a). At this pH, the amount of soluble iron (Fe^{3+}) decreased and the scavenging effect of H^{+} ions inhibited the $OH^{\bullet}$ formation (Lucas and Peres 2006).

Increasing pH above 4 inhibited both the regeneration of the active species Fe^{2+} and the formation of hydroxyl radicals (El-Desoky et al. 2010). Besides, this technique also involved hydroxyl radicals; presence of organic matter and alkalinity of WW promotes scavenging of $OH^{\bullet}$. This narrow pH range, catalyst recovery, temperature, hydrogen peroxide and target-compound concentrations are the deciding factors for performance of the process. Further, the efficiency of this method may be enhanced with UV irradiation (photo-Fenton) where more hydroxyl radicals are produced compared to Fenton. Photo-Fenton is generally unsuitable to wastewaters with high organic matter concentration, since the turbidity prevented the penetration of UV radiation. In these matrices, Fenton process was more efficient even though it had lower removal efficiency and mineralization. For un-controlled pH working conditions and easy catalyst recovery, immobilization of Fenton catalyst on a heterogeneous matrix would possibly be a step to the direction for future investigations.

Wastewater effluents with high chemical oxygen demand (COD) and low biodegradability can be treated efficiently with combined Fenton-SBR process (Wu et al., 2013). Biological treatment alone cannot readily remove the refractory compounds and their by-products and results in adsorption on the sludge flocs. Application of Fenton oxidation process as a pre-treatment step improved the removal of antibiotics from WW as seen in table 2.1.4. Independent optimization of each chemical and biological step is normally required for efficient designing of the technology. Various studies (DeWitte et al. 2008; Vasconcelos et al. 2009; Liu et al. 2012; Babić et al. 2013) showed that ciprofloxacin photolysis; transformation products (TPs) lost their fluorine atom whereas in ozonation and photo-Fenton, TPs retained fluorine atom. The abundance of intermediates products (toxicity) depended on pH of the treatments. Even though all these treatment mechanisms involved OH radical attack (O_3 radical for ozonation), the formed degradation products, and final toxicity of treated WW and efficiency of methods were different for the same antibiotic. This was mainly due to the difference in the physical-chemical parameters of the treatment (pH, initial antibiotic,O_3 and H_2O_2 concentration, NOM, time, pKa of TPs etc.)

Photocatalysis

Photocatalysis is investigated as AOPs for environmental applications to improve the biodegradability of antibiotic compounds. Wastewaters containing buffered compounds which are alkaline in nature scavenge the hydroxyl radicals. In this case, oxidation technologies with $OH^{\bullet}$ attack (UV/H_2O_2, O_3/H_2O_2, Fenton and photo-Fenton (homogenous photocatalysis) have limited applications.

To overcome these limitations, heterogeneous photocatalysis using semiconductor surfaces provided a fixed environment to initiate light induced redox reactions. Titanium dioxide (TiO_2) is the most widely accepted photocatalyst to detoxify polluted water, air, and soils. It has key advantages, such as readily available, inexpensive, non-toxic, chemically and mechanically stable, and commercially available in various crystalline forms. Considering the spectroscopic features, it exhibits strong oxidizing photoactivity in UV light. From a mechanistic point of view, absorption of photons with energy higher than the band gap (the area between valence and conduction bands) energy of the semiconductor generates valence band holes and conduction band electrons. These holes have a very high oxidation potential capable of generating hydroxyl radicals from the water molecules or hydroxide ions adsorbed on the semiconductor surface. The electrons can react with adsorbed molecular oxygen and reduce it to peroxide radicals (Fujishima et al. 2008).

Overall, semiconductor photocatalysis involves adsorption and oxidation of WW contaminants in adsorbed phase on catalyst surface and removal of products from the interface region. Critical operating parameters dictating performance are wavelength, radiation intensity, catalyst concentration, pH and water matrix. Solution pH is important as it decides the ionization state of the catalyst surface and consequently affects the extent of adsorption and degradation (Giraldo et al. 2010). Typical photocatalytic reactions mostly involve TiO_2 suspensions. To recover the catalyst from treated effluents, post-treatment with membrane filtration has been demonstrated (Xiao et al. 2010). The role of the membrane was to retain the used catalyst, the untreated pharmaceuticals and their by-products. And another alternative is that the catalyst may be immobilized on suitable support matrices (glass, silica gel, metal, ceramics, polymer, zeolite, alumina clays and AC) (Palominos et al. 2008; Miranda-Garcia et al. 2010) but this has the disadvantage of suffering from mass transfer limitation due to the reduction in specific surface area of catalyst.

Holes and electrons may undesirably recombine thus to prevent this, several approaches for TiO_2 modification have been proposed: doped-TiO_2 with metallic and nonmetallic species (Zaleska, 2008; Jo and Kim, 2009; Huo et al. 2011). Palominos et al. (2009) highlighted that pKa of the target compound and catalyst and pH of the treatment solution have significant role in degradation. At acidic pH (<3.3), both tetracyclines and titanium were positively charged and at high pH (>9.7), they were negatively charged leading to repulsive effects. Adsorption of the compound on catalyst surface was essential for degradation which was influenced by pKa and pH of the compound and the treatment solution.

Ultrasonication

Application of ultrasonication technology to wastewater treatment has been developed in recent times and this is also reflected in the smaller number of publications concerning antibiotic treatment. Sonochemical reactions are induced upon irradiation of a liquid sample with ultrasonic (typically in the range 20-1000 kHz) waves resulting in agitation leads to bubbles creation. Formation, growth, and implosive collapse of bubbles caused by cavitation produce intense local heating and high pressures, leads to the formation of highly reactive hydroxyl radicals. Cavitation serves as an effective means of concentrating the diffuse energy of sound (the conversion of the kinetic energy of liquid motion) as short-lived localized hot-spot. There are three potential sites for sonochemical reactions, namely: the cavitation bubble itself, the interfacial region between the bubble and the surrounding liquid and the solution bulk. According to these reaction sites, antibiotic contaminants with low solubility (at interfacial region) and volatile nature (in cavitation bubble itself) undergo fast sonochemical degradation. For organic compounds which are polar and difficult to volatilize, the reaction mainly occurs in the cavitation bubble surface layer or liquid within the body by free radical oxidation.

The most important factors that affect process efficiency are the frequency and intensity of ultrasound, nature of contaminant, bulk temperature, pH and the type of matrix (De Bel et al. 2009, 2011). Cavitation is a nucleated process; hence WW matrix composition is mainly important as large numbers of particulates serve as nuclei usually improving the performance. Sonolysis has proven to be an effective method for degrading organic effluents into less toxic. Guo et al. (2010) studied for fabrication of different additives which resulted in enhancement of the free radicals by easy generation of the cavities and additional oxidizing mechanisms in the system intensifying the degradation process. However, to obtain complete mineralization of the pollutants alone with ultrasound treatment is not economically acceptable (Torres et al. 2009). And also ultrasound is found to be more effective when used in combination with other treatment processes (Sangave and Pandit 2004). Combining ozone and/or hydrogen peroxide with ultrasound leads to higher degradation rates due to increased dissociation of ozone under the action of ultrasound. The operating frequency is crucial factor in deciding the synergism. The enhanced turbulence generated by ultrasound eliminates the mass transfer resistance, which is a major limiting factor for the application of ozone or hydrogen peroxide alone (Gogate 2008). According to De Bel et al. (2009), protonated ciprofloxacin is more susceptible to ultrasonic degradation and it is generally dependent on pH of the treatment method.

Elecrochemical processes

Electrochemical treatment of WW to remove toxic organic compounds is very interesting. It was considered as an effective and environmental friendly process due to its strong oxidizing ability, simplicity in operation, reliable performance for a wide variety of toxic organics. Generally, it involves redox reactions for degradation of waste through electron transfer between the electrode and the electrolyte or compounds in solution. In electrochemical process, direct oxidation can take place on the anode (Ti-based alloys, highly boron-doped diamond (BDD), Pt, graphite electrodes, carbon, and iridium and ruthenium oxide) surface through the anodic electron exchange. Indirect oxidation is mediated by the oxidants that are formed electrochemically. Such oxidants include chlorine, hypochlorite, ozone and hydrogen peroxide. The selection and process efficiency depends on the electrode material, electrolyte composition, and organic concentration.

Electrochemical oxidation processes are the most efficient to treat toxic wastewaters with high concentrations of antibiotics and COD yielding higher mineralization degree (Miyata et al. 2011). The oxidation efficiency of this AOP is enhanced by external addition of Fe^{2+} which catalyzes the degradation of electro generated H_2O_2 to hydroxyl radicals (Guinea et al. 2010).

Low Cost Activated Carbon

Near the beginning of 19th century, an overview of development showed that the applications of Activated Carbon (AC) was performed mainly for the purpose of dechlorination of chlorinated water and in treatment of water for removal of substances responsible for taste and odor (Sontheimer et al. 1988). For many decades, adsorption of natural organic matter (NOM) on AC appeared to be very effective and reliable method. The main problem with antibiotics is that they are difficult to mineralize completely by biological or conventional wastewater treatment plant processes. There are number of physical-chemical processes that have been used and great efforts were made to modify them to improve effectiveness of the method. Among them, adsorption on AC material is most prevalent technology for the removal of low concentration pollutants.

Currently, AC is very often utilized in the removal of various organic (antibiotics) and inorganic species from surface water, ground water and WW (Wilson et al., 2006; Budinova et al. 2008). Activated carbon used for adsorption finds wide applications in many areas especially in the environmental pollution control and is also used in various liquid and gas applications (Gaspard et al. 2007; Lemus et al. 2012). Among the possible techniques for wastewater treatments, the adsorption process is seen as one of the most efficient and low cost method for the treatment and removal of organic contaminants from WW. Materials utilized for adsorption are low cost and locally available, such as natural materials (Mendez-Diaz et al. 2010), agricultural wastes (Zhang et al. 2010; Torres-Perez et al. 2012) and industrial wastes (Ding et al. 2012).

Kinetics linked with adsorption of organic contaminants is affected by acidic/basic character (surface heterogeneity) and functional groups associated with AC, (Ruiz et al. 2010). Studies proved that adsorption capacity was enhanced by increasing the interaction between adsorbate and surface functional groups and also depended upon pH of the adsorbent (Putra et al. 2009). Adsorbent and adsorbate chemical properties, such as hydrophobicity and pH are very important to establish the adsorption mechanism on ACs (Mendez-Diaz et al. 2010). Apart from this, impact on removal efficiency also involves pore size and volume of adsorbate (Ding et al. 2012). Effect of textural and chemical characteristics of AC on adsorption are determined by heating method (Huang et al. 2011). Nevertheless, AC can just concentrate the antibiotics and eventually the antibiotic adsorbed is a hazardous waste which needs to be disposed in a secured landfill. Hence, AC is just a method of compartmentalization of antibiotics from the liquid medium to the solid phase, which again questions the effectiveness of the method.

Hybridization of Activated Carbon

The effects of combined technologies are suggested to be as an effective barrier to reduce the discharge of trace contaminants into the aqueous environment or water resources (Zhang et al. 2006). Combination of AC with other treatment methods has been known to improve the antibiotic removal efficiency. Ozonation of antibiotics prior to treating with AC can increase their removal rate, efficacy, reduce the concentration of dissolved organic matter (Reungoat et al. 2012) and reduce the quantity of AC required as compared to stand alone treatment (Rivera-Utrilla et al. 2010). Studies reported that integrating the AC with membrane or membrane bioreactor can gradually improve the removal of the more recalcitrant compounds (Zhang et al. 2006; Serrano et al. 2011; Nguyen et al. 2013). Pretreatment of antibiotics prior to AC addition changes the polarity, concentration, toxicity of parent compounds and improves the reactivity between the by-products and AC. Recent studies have reported outstanding performance of low cost carbon materials (Mendez-Díaz et al. 2010; Helia et al. 2012) for the removal of antibiotics, which is an economically attractive alternative for water treatment along with waste disposal and recycling of adsorbent (Mestre et al. 2009) where other methods often involve high resources and operational costs leading to their limited application.

Table 2.1.5 highlights physico-chemical properties of compound and treatment method affecting the degradation pathways. As described in the table 2.1.5, all studies on determination of degradation pathways of antibiotics were performed in water and not in wastewater. WW is a complex matter; it is very difficult to target one compound for 100% removal efficiency as other compounds are also susceptible to degradation (redox reactions) with the same removal method and decrease the efficiency of method for aHence, it is challenging for scientists to find out an optimized method for application of degradation pathway and removal efficiency in water and wastewater.

Enzymatic degradation

Microorganisms are essential components of the biosphere; they perform extreme biochemical transformation reactions. Mainly, they convert fixed carbon photo-synthetically, that supports the global carbon cycle. Environmental pollutants that are of concern today present challenges to the microbial enzymes that catalyze degradation of naturally occurring organic compounds. However, numerous bacterial and fungal species have been isolated for efficient environment pollutant detoxification. There is a great demand for investigating novel biological methods for wastewater treatment for better future. Enzymatic degradation processes have been explored as novel and eco-friendly method for the removal of contaminants from WW. Earlier plasmids and enzymes have been isolated from wastewater bioreactors for domestic and industrial waste bioprocess (Bramucci and Nagarajan 2006). So far, very limited research has been reported on biocatalytic degradation of antibiotics using enzymes. A foremost challenge for this treatment is to understand the biochemical pathways and enzymes used by microorganisms for remediation of contaminants. Recent studies have successfully applied white-rot fungi and their extracellular modifying enzymes for degradation of antibiotics and trace organic contaminants (Prieto et al. 2011; Yang et al. 2013). Hyeyoung and Youn-Kyoo (2010) developed a special method using glutathione S-transferases (GSTs) for enzymatic degradation of antibiotics. These enzymes are believed to be involved in the cellular detoxification of substrates and forming glutathione adducts that are further metabolized or transported out of the cell (Van Hylckama Vlieg et al. 1998, 1999). Recently, investigation has been started to understsand the toxicity and persistence of the by-products obtained by this method.

However, the applications of these enzymes in environmental remediation are limited due to their lack of bio-stability and that they are easily washed out. Biotechnological processes based on immobilization/encapsulation of the biocatalysts have developed rapidly particularly those using viable, metabolically active microorganisms to prevent washing-out and facilitate their reuse. Many authors studied the stability and activity of free and immobilized enzymes. Stability of the immobilized was higher than that of the free enzyme. But the degradation rates were higher for the free compared to the immobilized enzymes due to mass transfer limitations for immobilized enzymes (Sheldon, 2007; Christoph et al. 2012; Hommes et al. 2012). The solid supports for immobilization of enzymes must be biocompatible, constitute a protective micro-environment and have large specific surface area, negligible swelling, high stability and low price. In the past decades, porous inorganic materials, especially silica nanoparticles have been widely used as an enzyme carrier material (Hommes et al. 2012). Critical process parameters include pH, temperature and chemical properties of the compounds (Cabana et al. 2007). This treatment is cost-effective, eco-friendly and can be easily applied in existing wastewater treatment plants.

A combination of ultrasound and enzyme assisted biodegradation was studied where enzymatic degradation as pre-treatment was carried out to treat WW effluents. This combination strategy showed synergistic effect in COD reduction (Sangave and Pandit 2006). This method demands future development for wide range of applications in wastewater treatment.

Cost evaluation of ww treatment technologies

Traditionally, WW effluent disinfection was carried out by using chlorine because of its effectiveness, residual properties, and low cost. However, many studies have showed that residual chlorine reacts with organics matter to form toxic by-products (trihalomethanes and haloacetic acids). The carcinogenic properties of these disinfection by-products could adversely affect public health and aquatic life (Morris and McKay1975).

Researchers promoted alternate disinfection technologies, such as ozonation and UV disinfection which are already in use for disinfection of drinking water. Apart from toxic by-products, the cost of chlorination and dechlorination chemicals has dramatically increased to meet the new wastewater discharge limits. Specifically, UV is most often selected because the installation of UV is a more cost-effective; it requires less maintenance and is long term operation. World Health Organization estimated costs of UV treatmeny as US$ 0.02 per 1,000 liters of water at the community level (including the cost of electricity and consumables and the annualized capital cost of the unit) (WHO 2014). Trojan Technologies evaluated the cost of UV disinfection for a new wastewater plant in Brazil over chemical alternatives would save the municipality between $1.3 – $2 million dollars over a 20 year period (include capital cost, equipment installation & construction, chemicals, lamp and power and maintenance costs) (Trojan Technologies 2014). Ozonation thus becomes a cost effective option for wastewater disinfection by eliminating on-going chemical costs. Ozone can be produced on site using oxygen from the ambient air. Hence, this makes the use of ozone not cost- and energy-effective. Only electrical power is required for operation. Kyle Greene et al. (2007) reported the total cost associated with the ozonation as $ 0.56/ m3 (including only the power consumption). Even though relative cost of application of ozone is high compared to UV disinfection; the high reactivity of ozone with a wide range of substances (including antibiotics) will make it the most appropriate disinfection technique for achieving specific treatment goals.

Ricky Priambodo et al. (2011) calculated the cost (including the cost of electricity) of Fenton and photo-Fenton processes as \$0.07 and \$0.163, respectively in real wastewater treatment. In this treatment, chemical cost contributed 90% of the technique cost. Greentech environment protection technology reported operation cost of Active carbon absorption, Ozone oxidation method and Fenton Method as \$ 3 ~ 10, 6.25 ~ 8.75 and 2.5 ~ 6.25 respectively (GreenTec 2012). Daghrir et al. (2014) studied the removal of chlortetracycline from spiked municipal wastewater using a photoelectrocatalytic process and estimated operating cost as \$4.17 ± 0.0/ m^3. Biological treatment is generally less expensive than chemical oxidation; hence, the combined biological and chemical treatments may reduce the total cost of the treatment.

Cost evolution of each treatment method varies and also the treatment cost varies with different WWTPs as each WWTP receives different composition and concentrations of organic matter and contaminants. Cost of a treatment method is affected by many parameters mainly composition of waste, efficiency of method, initial setup cost, chemical cost, energy consumption, labor, time and scale of the waste to be treated. All these parameters vary with the method and treatment plant. Hence, it is difficult to fix and evaluate the cost of a treatment method. Apart from the economic benefit of a method, efficiency and toxicity of final effluent should be considered for selecting the treatment method.

So far, the reported cost evaluation of treatment methods was for surface water and synthetic wastewater and not for the real wastewater. And also the cost calculations were done mostly for COD removal and disinfection of a treatment method. No studies were done on the cost of WWTs specifically corresponding to emerging contaminants (antibiotics) removal and their efficacy and hence the precise cost calculation is difficult to present at this stage.

Conclusions and future outlook

Many studies have gathered information on quantity and toxicity of antibiotic residues and their metabolites, especially when attempting to reuse WW and dispose sludge in agricultural areas and landfills. Several investigators reported that antibiotics are difficult to be completely removed using conventional and, biological/physic-chemical treatment processes.

The presence and fate of antibiotics in WW in particular, has received significant attention during the last two decades. Antibiotics are designed to be biologically active at relatively low concentrations. Exposures to antibiotics in the environment even at low concentrations (detected) may have adverse effects either in the aquatic or terrestrial ecosystems. Concentration of antibiotic contaminants varies with matrix and mineralization is affected by the WW matrix composition (Michael et al. 2012). Hence, treatment at source may be a plausible option.

In the past two decades, AOPs have been explored as alternatives to traditional processes. And also AOPs are proven as efficient treatment methods for degrading recalcitrant, stable and toxic contaminants. However, the main drawback of AOPs is their higher cost in comparison with traditional treatments. Operating cost of AOP treatment to oxidize antibiotics is not only dependent upon the efficiency of a process but also the nature of the effluent and the treatment volume. Many authors studied the possibility of process integration to maximize treatment performance and to reduce operating cost. The concept of coupling AOPs as a pre-treatment stage has gained a lot of attention over the past several years (Comninellis et al. 2008). This approach is based on the facts that: (i) complete mineralization by AOPs alone is expensive and the end-products that are formed tend to be refractory; (ii) these end-products however can be degraded easily by biological treatment which may be less costly and more environmentally friendly than any other destructive treatment (Li et al. 2008; Balcioğlu and Ötker 2003). In these studies, results demonstrated that ozonation as a partial step in a combined treatment concept was a potential technique for biodegradability enhancement for WW effluents. As discussed earlier, Fenton’s oxidation is also most studied AOPs, this often applied to widen range of antibiotics and WW matrices, especially when combined with UV irradiation (photo-Fenton) (Elmolla and Chaudhuri 2009b). It was proved that integration of Fenton with biological treatment increased the biodegradability of refractory compounds (Wu et al. 2013). In these cases, maximum degradation accompanied by increased mineralization was achieved. This strategy is based on the knowledge that some substances reduce their toxicity when they are partially oxidized and become easily biodegradable thus reducing the cost.

Adsorption is another process that has been reported as an alternative to oxidation techniques, though not widely applied to the more prescribed antibiotics. This technique has the advantage of removing the antibiotic residues instead of producing potentially more dangerous metabolites. The use of ozonation as pre-treatment, with subsequent treatment by AC is also frequent (Reungoat et al. 2012). Studies (Serrano et al. 2011; Nguyen et al. 2013) showed that integrating the AC with membrane process improved the removal of the large number recalcitrant compounds. However, it has the disadvantage of producing a new and often hazardous residue.

Despite the fact that the combined methods are not a very common practice; nevertheless the combined processes would be the best solution for the treatment of effluents containing antibiotics. AOPs followed by biological treatment or by a membrane or by an adsorption process is the most usual combined process (Zhang et al. 2006; Alpatova et al. 2013; Sánchez Pérez et al. 2013). These methods are not normally used due to their complexity and impracticability of being used in a continuous mode.

Wastewater treatment methods deserve greater attention to enumerate the fate of antibiotics. These compounds retain their adverse properties even at minute concentrations and need extremely high conversions at this concentrations makes expensive. Future research should focus on hybrid technologies of AOPs followed by biological treatment or eco-friendly enzymatic treatment. And also thorough elucidation of the nature of degradation products is necessary for complete treatment.

Acknowledgement

The authors are sincerely thankful to the natural sciences and engineering research council of canada (discovery grant 355254 and strategic grant), and ministère des relations internationales du québec (coopération québec-catalanya 2012-2014) for financial support. The views or opinions expressed in this article are those of the authors.

References

Abellán, M.N., Gebhardt, W., and Schröder, H.F. (2008). "Detection and identification of degradation products of sulfamethoxazole by means of LC/MS and -MSn after ozone treatment." *Water. Sci. Technol.,* 58(9), 1803-1812.

Aksu, Z., and Tunc, O. (2005). "Application of biosorption for penicillin G removal: comparison with activated carbon." *Process. Biochem.,* 40, 831-847.

Alcock, R.E., Sweetman, A., and Jones, K.C. (1999). "Assessment of organic contaminant fate in wastewater treatment plants. I: Selected compounds and physicochemical properties." *Chemosphere,* 38, 2247-2262.

Alpatova, A.L., Davies, S.H., and Masten, S.J. (2013). "Hybrid ozonation-ceramic membrane filtration of surface waters: The effects of water characteristics on permeate flux and the removal of DBP precursors, dicloxacillin and ceftazidime." *Sep. Purif. Technol.,*107, 179-186.

Avisar, D., Lester, Y., and Mamane, H. (2010). "pH induced polychromatic UV treatment for the removal of a mixture of SMX, OTC and CIP from water." *J. Hazard. Mater.,* 175, 1068-1074.

Babić, S., Periša, M., and Škorić, I. (2013). "Photolytic degradation of norfloxacin, enrofloxacin and ciprofloxacin in various aqueous media." *Chemosphere*, 91, 1635-1642.

Balcioğlu Akmehmet, I., and Ötker, M. (2003). "Treatment of pharmaceutical wastewater containing antibiotics by O_3 and O_3/H_2O_2 processes." *Chemosphere,* 50, 85-95.

Batt, A.L., Bruce, I.B., and Aga, D.S. (2006). "Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges." *Environ. Pollut.,* 142, 295-302.

Bautitz, I.R., and Nogueira, R.F. (2007). "Degradation of tetracycline by photo-Fenton process-Solar irradiation and matrix effects." *J. Photoch. Photobio. A Chem.,* 187 (1), 33-39.

Ben, W., Qiang, Z., Pan, X., and Nie, Y. (2012). "Degradation of veterinary antibiotics by ozone in swine wastewater pretreated with sequencing batch reactor." *J. Environ. Eng.,* 138, 272-277.

Bramucci, M., and Nagarajan, V. (2006). "Bacterial communities in industrial wastewater bioreactors." *Curr. Opin. Microbiol.,*9, 275-278.

Budinova, T., Petrov, N., Parra, J., and Baloutzov, V. (2008). "Use of an activated carbon from antibiotic waste for the removal of Hg (II) from aqueous solution." *J. Environ. Manage.,* 88, 165-172.

Cabana, H., Jiwan, J.L., Rozenberg, R., Elisashvili, V., Penninckx, M., Agathos, S.N., and Jones, J.P. (2007). "Elimination of endocrine disrupting chemicals nonylphenol and bisphenol A and personal care product ingredient triclosan using enzyme preparation from the white rot fungus Coriolopsis polyzona." *Chemosphere.,* 67 (4), 770-778.

Calza, P., Medana, C., Carbone, F., Giancotti, V., and Baiocchi, C. (2008). "Characterization of intermediate compounds formed upon photoinduced degradation of quinolones by high-performance liquid chromatography/high-resolution multiple-stage mass spectrometry." *Rapid. Commun, Mass Spectrom.,*22(10), 1533-1552.

Christoph, A.G., Gregor, H., and Philippe, F.X.C. (2011). "Removal of phenolic pollutants from municipal wastewater through immobilized laccase enzymes." *Environ. Eng. Manage. J.* 11.

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N., and Kroiss, H. (2005). "Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants." *Water Res.,* 39: 4797-4807.

Comninellis, C., Kapalka, A., Malato, S., Parsons, S.A., Poulios, I., and Mantzavinos, D. (2008). "Advanced oxidation processes for water treatment: advances and trends for R&D." *J. Chem. Technol. Biotechnol.* 83, 769-776.

Conly, J. (2001). "Antimicrobial resistance in Canada: An update on activities of the Canadian Committee on Antibiotic Resistance." *Can. J. Infect. Dis.,* 12, 215–217.

Darlymple, O.K., Yeh, D.H., and Trotz, M.A. (2007). "Removing pharmaceuticals and endocrine-disrupting compounds from wastewater by photocatalysis." *J. Chem. Technol. Biotechnol.* 82, 121-134.

Davies, J. (1996). "Origins and Evolution of Antibiotic Resistance." *Microbiologia,*12 (1), 9-16.

Davison, J. (1999). "Genetic Exchange between Bacteria in the Environment." *Plasmid,* 42, 73-91.

De Bel, E., Dewulf, J., Witte, B.D., Van Langenhove, H., and Janssen, C. (2009). "Influence of pH on the sonolysis of ciprofloxacin: Biodegradability, ecotoxicity and antibiotic activity of its degradation products." *Chemosphere*, 77, 291-295.

De Bel, E., Janssen, C., De Smet, S., Van Langenhove, H., and Dewulf, J. (2011). "Sonolysis of ciprofloxacin in aqueous solution: Influence of operational parameters." *Ultrason, Sonochem.,*18, 184-189.

DeWitte, B., Dewulf, J., Demeestere, K., Van De Vyvere, V., De Wispelaere, P., and Van Langenhove, H. (2008). "Ozonation of ciprofloxacin in water: HRMS identification of reaction products and pathways." *Environ. Sci. Technol.,* 42(13),4889-4895.

Ding, R., Zhang, P., Seredych, M., and Bandosz, T.J. (2012). "Removal of antibiotics from water using sewage sludge- and waste oil sludge-derived adsorbents." *Water Res.,* 46, 4081-4090.

Dorival-García, N., Zafra-Gomez, A., Navalón, A., Gonzalez, J., and Vílchez, J.L. (2013). "Removal of quinolone antibiotics from wastewaters by sorption and biological degradation in laboratory-scale membrane bioreactors." *Sci. Total. Environ.,*442, 317-328.

El-Desoky, H.S., Ghoneim, M.M., El-Sheikh, R., and Zidan, N.M. (2010). “Oxidation of Levafix CA reactive azo-dyes in industrial wastewater of textile dyeing by electro-generated Fenton’s reagent.” *J. Hazard. Mater.,*175, 858-865.

Elmolla, E., and Chaudhuri, M. (2009a). “Optimization of Fenton process for treatment of amoxicillin, ampicillin and cloxacillin antibiotics in aqueous solution.” *J. Hazard. Mater.,*170 (2-3), 666-672.

Elmolla, E.S., and Chaudhuri, M. (2009b). “Improvement of biodegradability of synthetic amoxicillin wastewater by photo-Fenton process.” *World. Appl. Sci. J.,* 5, 53-58.

Elmolla, E.S., Chaudhuri, M., and Mohamed, M.E. (2010). “The use of artificial neural network (ANN) for modeling of COD removal from antibiotic aqueous solution by the Fenton process.” *J. Hazard. Mater.,*179, 127-134.

Ewa, A., wojciech, b., Justnya, Z., and Andrzej, S. (2010). “Effect of $FeCl_3$ on sulfonamide removal and reduction of antimicrobial activity of waste water in a photocatalytic process with TiO_2.” *Appl. Catal. B.,* 126, 29-38.

Fernando, J.B., Almudena, A., Garcia-Araya, J.F., and Ana, O. (2008). “Ozone and photocatalytic processes to remove the antibiotic sulfamethoxazole from water.” *Water Res.,* 42, 3799-3808.

Fuentefria, D.B., Ferreira, A.E., and Corção, G. (2011). “Antibiotic-resistant Pseudomonas aeruginosa from hospital wastewater and superficial water: Are they genetically related?” *J. Environ. Manage.,*92 (1), 250-255.

Fujishima, A., Zhang, X., and Tryk, D.A. (2008). “TiO2 photocatalysis and related surface phenomena.” *Surf. Sci. Rep.,* 63, 515-582.

García-Galána, M.J., Díaz-Cruz, M.S., and Barceló, D. (2012). “Kinetic studies and characterization of photolytic products of sulfamethazine, sulfapyridine and their acetylated metabolites in water under simulated solar irradiation.” *Water Res.,* 46 (3), 711-722.

Gaspard, S., Altenor, S., Dawson, E.A., Barnes, P.A., and Ouensanga, A. (2007). “Activated carbon from vetiver roots: Gas and liquid adsorption studies.” *J. Hazard. Mater.,* 144 (1-2), 73-81.

Giraldo, A.L., Peñuela, G.A., Torres-Palma, R.A., Pino, N.J., Palominos, R.A., and Mansilla, H.D. (2010). “Degradation of the antibiotic oxolinic acid by photocatalysis with TiO_2 in suspension.” *Water Res.,* 44 (18), 5158-5167.

Gogate, P.R. (2008). “Treatment of wastewater streams containing phenolic compounds using hybrid techniques based on cavitation: A review of the current status and the way forward.” *Ultrason. Sonochem.,*15,1-15.

Gómez-Ramos Mdel, M., Mezcua, M., Agüera, A., Fernández-Alba, A.R., Gonzalo, S., Rodríguez, A., and Rosal, R. “Chemical and toxicological evolution of the antibiotic sulfamethoxazole under ozone treatment in water solution.” *J. Hazard. Mater.* 192(1), 18-25.

Goossens, H., Coenen, S., Costers, M., De Corte, S., De Sutter ,A., Gordts, B., Laurier ,L., and Struelens, M. (2008). “Achievements of the belgian antibiotic policy coordination committee (BAPCOC).” *Euro. Surveill.* 13(46), pii: 19036.

GreenTec, 2012, available at http://www.biogreentec.com.tw/pages/en/theory.html#topic3.

Guinea, E., Brillas, E., Centellas, F., Cañizares, P., Rodrigo, M.A., and Sáez, C. (2009). “Oxidation of enrofloxacin with conductive-diamond electrochemical oxidation, ozonation and Fenton oxidation: a comparison.” *Water Res.,* 43 (8), 2131-2138.

Guinea, E., Garrido, J.A., Rodríguez, R.M., Cabot, P.L., Arias, C., Centellas, F., and Brillas, E. (2010). “Degradation of the fluoroquinolone enrofloxacin by electrochemical advanced oxidation processes based on hydrogen peroxide electrogeneration.” *Electrochim. Acta.,*55 (6), 2101-2115.

Guo, W., Shi, Y., Wang, H., Yang, H., and Zhang, G. (2010). “Intensification of sonochemical degradation of antibiotics levofloxacin using carbon tetrachloride.” *Ultrason. Sonochem.,* 17 (4), 680-684.

Hapeshi, E., Fotiou, I., and Fatta-Kassinos, D. (2013). “Sonophotocatalytic treatment of ofloxacin in secondary treated effluent and elucidation of its transformation products.” *Chem. Eng. J.,* 224, 96-105.

Hirsch, R., Ternes, T., Haberer, K., and Kratz, K.L. (1999). “Occurrence of antibiotics in the aquatic environment.” *Sci. Total. Environ.,* 225(1-2), 109-118.

Helia, M.C., Cibele, C.C.A., Adriana, S.F., and Leandro, S.O. (2012). “Evaluation of the performance of an agricultural residue-based activated carbon aiming at removal of phenylalanine from aqueous solutions.” *Food. Sci. Technol. LEB.,* 49(1), 155-161.

Homem, V., and Santos, L. (2011). “Degradation and removal methods of antibiotics from aqueous matrices.” *J. Environ. Manage.* 92(10), 2304-2347.

Hommes, G., Gasser, C.A., Howald, C.B., Goers, R., Schlosser, D., Shahgaldian, P., and Corvini, P.F. (2012). “Production of a robust nanobiocatalyst for municipal wastewater treatment.” *Bioresour. Technol.,*115, 8-15.

Huang, C.H., Renew, J.E., Pinkston, K., and Sedlak, D.L. (2011). “Occurrence and fate of antibiotic compounds in municipal wastewater and animal waste.” *Water. Environ. Res.,* 12, 686-697.

Huang, Li., Sun, Y., Wang, W., Yue, Q., and Yang, T. (). “Comparative study on characterization of activated carbons prepared by microwave and conventional heating methods and application in removal of oxytetracycline (OTC).” *Chem. Eng. J.,* 171(3),1446-1453.

Huo, P., Lu, Z., Wang, H., Pan, J., Li, H., Wu, X., Huang, W., and Yan, Y. (2011). “Enhanced photodegradation of antibiotics solution under visible light with Fe^{2+}/Fe^{3+} immobilized on TiO_2/fly-ash cenospheres by using ions imprinting technology.” *Chem Eng J.,* 172(2-3), 615-622.

Hyeyoung, P., and Youn-Kyoo, C. (). 2010 “Evaluation of the biodegradation feasibility of antibiotics by three bacteria involving glutathione S-transferases.” *Can J Civil Eng* ., 37, 814-819.

Idris, Y., Aissani-Benissad, F., Florence, F., and Abdeltif, A. (2013). “Removal of tetracycline hydrochloride from water based on direct anodic oxidation (Pb/PbO_2 electrode) coupled to activated sludge culture.” *Chem Eng J*., 221, 418-425.

Jiao, S., Zheng, S., Yin, D., Wang, L., and Chen, L. (2008). “Aqueous oxytetracycline degradation and the toxicity change of degradation compounds in photoirradiation process.” *J. Environ. Sci* ., 20(7), 806-813.

Jin, X., Peldszus, S., and Huck, P.M. (2012). “Reaction kinetics of selected micropollutants in ozonation and advanced oxidation processes.” *Water Res*., 46(19), 6519-6530.

Jo, W.K., and Kim, J.T. (2009). “Application of visible-light photocatalysis with nitrogen-doped or unmodified titanium dioxide for control of indoor-level volatile organic compounds.” *J. Hazard. Mater.,* 164(1), 360-366.

Karthikeyan, K.G., and Meyer, M.T. (2006). “Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA.” *Sci. Total Environ.* 36(1-3), 196-207.

Kemper, N., Färber, H., Dirk, S., and Joachim, K. (2008). “Analysis of antibiotic residues in liquid manure and leachate of dairy farms in Northern Germany.” *Agric. Water Manage*., 95(11), 1288-1292.

Khan, M.H., Bae, H., and Jung, J.Y. (2010). “Tetracycline degradation by ozonation in the aqueous phase: proposed degradation intermediates and pathway.” *J. Hazard. Mater.,*181(1-3), 659-65.

Klavarioti, M., Mantzavinos, D., and Kassinos, D. (2009). “Removal of residual pharmaceuticals from aqueous systems by advanced oxidation processes.” *Environ. Int*., 35(2), 402-417.

Kolpin, D.W., Skopecb, M., Meyer, M.t., Furlong, E.T., and Zaugg, S.D. (2004). “Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions.” *Sci. Total Environ.,* 328(1-3), 119-130.

Kuixiao, L., Ayfer, Y., Min, Y., Schulte-Hostede, S., and Ming, H.W. (2008). “Ozonation of oxytetracycline and toxicological assessment of its oxidation by-products. *Chemosphere.,* 72(3), 473-478.

Kümmerer, K. (2009). "The presence of pharmaceuticals in the environment due to human use--present knowledge and future challenges. *J. Environ. Manage.,* 90(8), 2354-2366.

Kümmerer, K., Alexy, R., Hüttig, J., and Schöll. A. (2004). "Standardized tests fail to assess the effects of antibiotics on environmental bacteria." *Water Res.,* 38(8), 2111-2116.

Kyle Greene, Mike Zelen, Harold Kinasewich, Chris Kinasewich, Todd Webb. (2007). "Ozone based wastewater treatment solutions".

Larsson, D.G.J. (2008). "Drug Production Facilities - An overlooked discharge source for pharmaceuticals to the environment." *Pharmaceuticals in the Environment.* Kummerer, K. Eds., Springer, Germany, 37-42.

Lee, H., Lee, E., Lee, C.H., and Lee, K. (2011). "Degradation of chlorotetracycline and bacterial disinfection in livestock wastewater by ozone-based advanced oxidation." *J. Ind. Eng. Chem*., 17(3), 468-473.

Lemus, J., Martin-Martinez, M., Palomar, J., Gomez-Sainero, L., Gilarranz, M.A., and Rodriguez, J.J. (2012). "Removal of chlorinated organic volatile compounds by gas phase adsorption with activated carbon." *Chem. Eng. J.,*211-212, 246-254.

Lester, Y., Avisar, D., and Mamane. H. (2011). "Removal of pharmaceuticals using combination of UV/H_2O_2/O_3 advanced oxidation process." *Water. Sci, Technol.*

Le-Minh, N., Khan, S.J., Drewes, J.E., and Stuetz, R.M. (2010). "Fate of antibiotics during municipal water recycling treatment processes." *Water Res.,* 44(15), 4295-4323.

Li, K., Yediler, A., Yang, M., Schulte-Hostede, S., and Wong, M.H. (2008). "Ozonation of oxytetracycline and toxicological assessment of its oxidation by-products." *Chemosphere.,*72(3), 473-478.

Lin, A.Y., Lin, C.F., Chiou, J.M., and Hong, P.K. (2009). "O_3 and O_3/H_2O_2 treatment of sulfonamide and macrolide antibiotics in wastewater." *J Hazard Mater.,* 171(1-3), 452-458.

Liu, C., Nanaboina, V., Korshin, G.V., and Jiang, W. (2012). “Spectroscopic study of degradation products of ciprofloxacin, norfloxacin and lomefloxacin formed in ozonated wastewater.” *Water Res.,*46(16), 235-246.

Lucas, M.S., and Peres, J.A. (2006). “Decolorization of the azo dye Reactive Black 5 by Fenton and photo-Fenton oxidation.” *Dyes. Pigm.,* 71(3), 235-243.

Martinez, J.L. (2009). “Environmental pollution by antibiotics and by antibiotic resistance determinants.” *Environ. Pollut.,*157 (11), 2893-2902.

McDonald’s Global Antibiotics Policy, 2003. “McDonald’s Global Policy on antibiotic use in food animals”.

Méndez-Díaz, J.D., Prados-Joya, G., Rivera-Utrilla, J., Leyva-Ramos, R., Sánchez-Polo, M., Ferro-García, M.A., and Medellín-Castillo, N.A. (2010). “Kinetic study of the adsorption of nitroimidazole antibiotics on activated carbons in aqueous phase.” *J. Colloid. Interface. Sci.,* 345(2), 481-490.

Mestre, A.S., Pires, J., Nogueira, J.M., Parra, J.B., Carvalho, A.P., and Ania, C.O. (2009). “Waste derived activated carbons for removal of ibuprofen from solution: role of surface chemistry and pore structure.” *Bioresour Technol.,* 100 (5), 1720-1726.

Michael, I., Hapeshi, E., Osorio, V., Perez, S., Petrovic, M., Zapata, A., Malato, S., Barceló, D., Fatta-Kassinos, D. (2012). “Solar photocatalytic treatment of trimethoprim in four environmental matrices at a pilot scale: Transformation products and ecotoxicity evaluation.” *Sci. Total Environ.,* 430, 167-173.

Mira, P., Susana, G., and Damià, B. (2013). “Analysis and removal of emerging contaminants in wastewater and drinking water.” *TrAC, Trends Anal. Chem.,* 22(10), 685-696.

Miranda-Garcia, N., Ignacio, Maldonadoa, M., Coronado, J.M., and Malatoa, S. (2010). “Degradation study of 15 emerging contaminants at low concentration by immobilized TiO_2 in a pilot plant.” *Catal. Today* , 151(1-2), 107-113.

Miyata, M., Ihara, I., Yoshid, G., Toyod, K., and Umetsu, K. (2011). “Electrochemical oxidation of tetracycline antibiotics using a Ti/IrO_2 anode for wastewater treatment of animal husbandry.” *Water Sci. Technol.,* 63(3), 456-461.

Morris, J.C., McKay, G. (1975). “Formation of halogenated organics by chlorination of water supplies: a review”. Office of Research and Development, U.S. Environmental Protection Agency, Washington.

Nguyen, L.N., Hai, F.I., Kang, J., Price, W.E., and Nghiem, L.D. (2013). “Coupling granular activated carbon adsorption with membrane bioreactor treatment for trace organic contaminant removal: Breakthrough behaviour of persistent and hydrophilic compounds.” *J. Environ. Manage.,* 119, 173-181.

Novo, A., Andre, S., Viana, P., Nunes, O.C., and Manaia, C.M. (2013). “Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater.” *Water Res.,* 47(5), 1875-1887.

Ocsana, O., Florina, C., Maria, L.S., Dumitru, R., Ulo, N., and Lucian. C. (2013). “Influence of nine antibiotics on key secondary metabolites and physiological characteristicsin *Triticum aestivum*: Leaf volatiles as a promising new tool to assess toxicity.” *Ecotox. Environ. Safe.,* 87, 70-79.

Oller, I., Malato, S., and Sánchez-Pérez, J.A. (2011). “Combination of advanced oxidation processes and biological treatments for wastewater decontamination.” *Sci Total Environ.,* 409(20), 4141-4166.

Ostermann, A., Siemens, J., Welp, G., Xue, Q., Lin, X., Liu, X., Amelung, W. (2013). “Leaching of veterinary antibiotics in calcareous Chinese croplands.” *Chemosphere*., 91(7), 928-934.

Palominos, R.A., Mondaca, M.A., Giraldo, A., Peñuela, G., Pérez-Moya, M., Mansilla, H.D. (2009). “Photocatalytic oxidation of the antibiotic tetracycline on TiO_2 and ZnO suspensions.” *Catalysis Today,* 144(1-2), 100-105

Palominos, R.A., Mora, A., Mondaca, M.A., Pérez-Moya, M., Mansilla, H.D. (2008). “Oxolinic acid photo-oxidation using immobilized TiO_2.” *J. Hazard. Mater.,*158(2-3), 460-464.

Prados-Joya, G., Sanchez-Polo, M., Rivera-Utrilla, J., and Ferro-garcia, M. (2011). “Photodegradation of the antibiotics nitroimidazoles in aqueous solution by ultraviolet radiation”. *Water Res.,* 45(1), 393-403.

Prieto, A., Möder, M., Rodil, R., Adrian, L., and Marco-Urrea, E. (2011). “Degradation of the antibiotics norfloxacin and ciprofloxacin by a white-rot fungus and identification of degradation products.” *Bioresour. Technol.* 102(23), 10987-10995.

Putra, E.K., Pranowo, R., Sunarso, J., Indraswati, N., and Ismadji, S. (2009). “Performance of activated carbon and bentonite for adsorption of amoxicillin from wastewater: Mechanisms, isotherms and kinetics.” *Water Res.,* 43(9), 2419-2430.

Renato, F.D., Otidene, R., and Allan Kardec, R.T. (2010). “Anderson SMS, Lins da Silva V. Direct UV photolysis of propranolol and metronidazole in aqueous solution.” *Chem. Eng. J.,* 158(2), 143-147.

Reungoat, J., Escher, B.I., Macova, M., Argaud, F.X., Gernjak, W., and Keller, J. (2012). “Ozonation and biological activated carbon filtration of wastewater treatment plant effluents.” *Water Res*., 46 (3), 863-872.

Ricky Priambodo, Yu-Jen Shih, Yu-Jen Huang and Yao-Hui Huang. (2011). “Treatment of real wastewater using semi batch (Photo)-Electro-Fenton method”. *Sustain. Environ. Res.,* 21(6), 389–393

Rivera-Utrilla, J., Sánchez-Polo, M., Prados-Joya, G., Ferro-García, M.A., and Bautista-Toledo, I. (2010). “Removal of tinidazole from waters by using ozone and activated carbon in dynamic regime”. *J. Hazard. Mater.,* 174(1-3), 880-886.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., and Fatta-Kassinos, D. (2013). “Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment.” *Sci. Total. Environ.,* 447, 345-360.

Rodrigues-Silva, C., Maniero, M.G., Rath, S., and Guimarães, J.R. (2013). "Degradation of flumequine by the Fenton and photo-Fenton processes: evaluation of residual antimicrobial activity." *Sci Total Environ.,*445-446, 337-46.

Ruiz, B., Cabrita, I., Mestre, A.S., Parra, J.B., Pires, J., Carvalho, A.P., and Ania, C.O. (2010). "Surface heterogeneity effects of activated carbons on the kinetics of paracetamol removal from aqueous solution." *Appl. Surf. Sci.,* 256(17), 5171-5175.

Saghafinia, M.S., Emadian, S.M., Vossoughi, M. (2011). "Performances Evaluation of Photo-Fenton Process and Sonolysis for the Treatment of Penicillin G Formulation Effluent." *Procedia. Environ. Sci.,* 8, 202-208.

Sánchez Pérez, J.A., Román Sánchez, I.M., Carra, I., Cabrera Reina, A., Casas López, J.L., and Malato, S. (2013). "Economic evaluation of a combined photo-Fenton/MBR process using pesticides as model pollutant. Factors affecting costs. " *J. Hazard. Mater.,* 244-245, 195- 203.

Sánchez-Polo, M., Rivera-Utrilla, J., Prados-Joya, G., Ferro-García, M.A., and Bautista-Toledo, I. (2008). "Removal of pharmaceutical compounds, nitroimidazoles, from waters by using the ozone/carbon system." *Water Res.,* 42(15), 4163-4171.

Sanchez-Prado, L., Barro, R., Garcia-Jares, C., Llompart, M., Lores, M., Petrakis, C., Kalogerakis, N., Mantzavinos, D., and Psillakis, E. (2008). "Sonochemical degradation of triclosan in water and wastewater." *Ultrason. Sonochem.,* 15(5), 689-694.

Sangave, P.C., and Pandit, A.B. (2004). "Ultrasound pre-treatment for enhanced biodegradability of the distillery wastewater." *Ultrason. Sonochem.,* 11(3-4), 197-203.

Sangave, P.C., and Pandit, A.B. (2006). "Ultrasound and enzyme assisted biodegradation of distillery wastewater." *J. Environ. Manage.,* 80(1), 36-46.

Sarmah, A.K., Meyer, M.T., and Boxall, A.B. (2006). “A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment.” *Chemosphere,* 65(5), 725-59.

Serpone, N., Horikoshi, S., and Emeline, A.V. (2010). “Microwaves in advanced oxidation processes for environmental applications. A brief review.” *J. Photochem. Photobiol., C*., 11(2-3),114-131.

Serrano, D., Suárez, S., Lema, J.M., and Omil F. (2011). “Removal of persistent pharmaceutical micropollutants from sewage by addition of PAC in a sequential membrane bioreactor.” *Water Res.,* 45(16), 5323-5333.

Sheldon, R.A. (2007). “Enzyme immobilization: The Quest for optimum performance.” *Adv. Synth. Catal.,* 349(8-9), 1289-1307.

Sontheimer, H., Crittenden, J.C., and Summers, R.S. (1988). “Activated carbon for water treatment.” 2nd edn, Forschungstelle Engler-Bunte-Institute, Universitat Karlsruhe, Germany.

Torres, R.A., Mosteo, R., Petrier, C., and Pulgarin, C. (2009). Experimental design approach to the optimization of ultrasonic degradation of alachlor and enhancement of treated water biodegradability.” *Ultrason. Sonochem.,* 16(3), 425-430.

Torres-Pérez, J., Gérente, C., and Andrès, Y. (2012). “Sustainable Activated Carbons from Agricultural Residues Dedicated to Antibiotic Removal by Adsorption.” *Chinese. J. Chem. Eng.,* 20(3), 524-529.

Trojan Technologies, 2014, available at http://www.trojanuv.com/resources/trojanuv/Introduction_to_TrojanUV_Wastewater___Drinking_Water_Disinfection.pdf.

Trovó, A.G., Melo, S.A.S., and Nogueira, R.F.P. (2008). “Photodegradation of the pharmaceuticals amoxicillin, bezafibrate and paracetamol by the photo-Fenton process - application to sewage treatment plant effluent.” *J. Photochem. Photobiol. A.,* 198(2-3), 215-220.

Trovó, A.G., Nogueira, R.F., Agüera, A., Fernandez-Alba, A.R., and Malato, S. (2011). "Degradation of the antibiotic amoxicillin by photo-Fenton process--chemical and toxicological assessment." *Water Res.,* 45(3), 1394-402.

Van Hylckama Vlieg, J.E., Kingma, J., Kruizinga, W., and Janssen, D.B. (1999). "Purification of a glutathione-S-transferase and a glutathione conjugate-specific dehydrogenase involved in isoprene metabolism in *Rhodococcus* sp. strain AD45." *J. Bacteriol.,* 181(7), 2094-2101.

Van Hylckama Vlieg, J.E., Kingma, J., Van de Wijngaard, A.J., and Janssen, D.B. (1998). "A glutathione S-transferase with activity towards *cis* -1,2-dichloroepoxythane is involved in isoprene utilization by *Rhodococcus* sp. Strain AD45." *Appl. Environ. Microbiol.,* 64(8), 2800-2805.

Vasconcelos, T.G., Henriques, D.M., König, A., Martins, A.F., Kümmerer, K. (2009). "Photo-degradation of the antimicrobial ciprofloxacin at high pH: identification and biodegradability assessment of the primary by-products." *Chemosphere,* 76(4), 487-493.

Vaz-Moreira, I., Nunes, O.C., and Manaia, C.M. (2012). "Diversity and antibiotic resistance in Pseudomonas spp. from drinking water." *Sci Total Environ.,* 426, 366-374.

Vignesh, S., Muthukumar, K., and James, R.J. (2012). "Antibiotic resistant pathogens versus human impacts: A study from three eco-regions of the Chennai coast, southern India." *Mar. Pollut. Bull.,* 64 (4), 790-800.

WHO, 2014, available at http://www.who.int/water_sanitation_health/dwq/wsh0207/en/index4.html

Wilson, K., Yang, H., Seo, C.W., and Marshall, W.E. (2006). "Select metal adsorption by activated carbon made from peanut shells." *Bioresour. Technol.,* 97(18), 2266-2270.

Wu, D., Wang, W., Guo, Q., and Shen, Y. (2013). "Combined Fenton-SBR process for bamboo industry wastewater treatment." *Chem. Eng. J.,* 214, 278-284.

Xiao-Huan, W., and Angela Yu-Chen, L. (2012). “Phototransformation of Cephalosporin Antibiotics in an Aqueous Environment Results in Higher Toxicity.” *Environ. Sci. Technol.,* 46(22), 12417-12426.

Xiao, Y.T., Xu, S.S., Li, Z.H., An, X.H., Zhou, L., Zhang, Y.L., and Shiang, F.Q. (2010). “Progress of applied research on TiO_2 photocatalysis-membrane separation coupling technology in water and wastewater treatments.” *Chin. Sci, Bull.,* 55(14), 1345-1353.

Yan, W., Hui, Z., Lu, C., Sheng, W., and Daobin, Z. (2012). “Ozonation combined with ultrasound for the degradation of tetracycline in a rectangular air-lift reactor. *Sep. Purif. Technol.,* 84, 138-146.

Yang, S., Hai, F.I., Nghiem, L.D., Price, W.E., Roddick, F., Moreira, M.T., and Magram, S.F. (2013). “Understanding the factors controlling the removal of trace organic contaminants by white-rot fungi and their lignin modifying enzymes: A critical review.” *Bioresour. Technol.,* 41, 97-108.

Yuan, F., Hu, C., Hu, X., Wei, D., Chen, Y., and Qu, J. (2011). “Photodegradation and toxicity changes of antibiotics in UV and UV/H_2O_2 process.” *J. Hazard. Mater.* 185(2-3), 1256-1263.

Zaleska, A. (2008). “Doped-TiO_2: A Review. Recent Patents on Engineering.” 2, 157-164.

Zhang, J., Lidietta, G., Enrico, D. (2006). “Study of a hybrid process combining PACs and membrane operations for antibiotic wastewater treatment.” *Desalination,* 194(1-3), 101-107.

Zhang, T., Walawender, W.P., and Fan, L.T. (2010). “Grain-based activated carbons for natural gas storage.” *Bioresour. Technol.,*101(6), 1983–1991.

Table 2.1.1 Classification of antibiotics based on the chemical structure, mode of action and the possible partitioning in wastewater treatment plant

Class	Mechanism of action	Examples	Property of compounds effecting its distribution in wastewater
B-lactam antibiotics	Prevents cell wall synthesis by binding to enzymes called penicillin binding proteins (PBPs)		
Penicillins		Penicillin V, Methicillin, Ampicillin, Amoxicillin, Piperacillin, Ticarcillin, and others	LogP is > 1 except Piperacillin <1 more partitioning in wastewater sludge
Cephalosporins		**1^{st} Generation** Cefacetrile, Cefadroxil, Cephalexin **2^{nd} Generation** Cefaclor, Cefonicid, Cefuroxime **3^{rd} Generation** Cefdaloxime, Cefdinir , Cefixime **4^{th} Generation** Cefclidine, Cefepime, Cefluprenam **5^{th} Generation** Ceftobiprole, Ceftaroline	LogP is ≤ 1 more partitioning in wastewater
Carbapenems		Imipenem, Doripenem, Meropenem, Ertapenem	LogP is ≤ -0.2 more partitioning in wastewater
Macrolides	Inhibit bacterial protein synthesis	Azithromycin, Erythromycin, Clarithromycin, Roxithromycin, Telithromycin	LogP is ≥ 3 more partitioning in wastewater sludge
Tetracyclines	Inhibit bacterial protein synthesis	Demeclocycline , Doxycycline, Minocycline, Monodox, Oxytetracycline, Chlortetracycline, Tetracycline, Tigecycline	LogP is ≤ 1 more partitioning in wastewater but complexation ability of these compounds with metals makes insoluble and retain in the sludge
Quinolones	Inhibit bacterial DNA synthesis	**1^{st} Generation** Flumequine, Nalidixic acid, Oxolinic acid. **2^{nd} Generation** Ciprofloxacin, Lomefloxacin, Nadifloxacin, Norfloxacin, Ofloxacin. **3^{rd} Generation** Gatifloxacin, Levofloxacin, Temafloxacin, Tosufloxacin **4^{th} Generation** Clinafloxacin, Gemifloxacin, Sitafloxacin	LogP is ≥ 1 more partitioning in wastewater sludge

Sulfonamides	Folic Acid synthesis inhibitors	Sulfamethizole, Sulfamethoxazole, Sulfisoxazole	LogP is < 0 more partitioning in wastewater
Aminoglycosides	Inhibit bacterial protein synthesis	Amikacin, Gentamicin, Kanamycin, Neomycin, Streptomycin Tobramycin	LogP is ≤ -3.0 more partitioning in wastewater
Imidazoles	Inhibit bacterial DNA synthesis (Metronidazole) and alter the cell membrane permeability	Metronidazole, tinidazole, clotrimazole, miconazole, econazole, ornidazole, ketoconazole	LogP is ≥ 5 more partitioning in wastewater sludge except for Metronidazole, Tinidazole LogP is < 0
Lincosamides	Inhibit bacterial protein synthesis	Clindamycin, Lincomycin	LogP range in between – 1 ≤ 0 ≥ 2 Partitioning in between wastewater and sludge
Peptides	Inhibit bacterial cell wall synthesis	Vancomycin, Teicoplanin Bacitracin, Polymyxin B Telavancin	LogP ≤ -2 more partitioning in wastewater
Others	Disrupt the bacterial protein biosynthesis	Chloramphenicol, Viomycin, Capreomycin, Linezolid	…

Table 2.1.2 A brief summary of effect of different matrices on degradation of antibiotics by AOPs

Antibiotic	**Matrix type**	**Treatment**	**Analytical Methods**	**Summary of results**	**Reference**
Trimethoprim	Demineralized water, simulated natural freshwater, simulated wastewater and real effluent	Solar photocatalysis	UPLC-QToF/MS HPLC–UV-DAD	- Photodegradation and mineralization were decreased (DW>SW>SWW>RE) as anions (Cl^-, SO_4^{-2}, DOC) and salt content increases (DW< SW<SWW<RE). - Organic and inorganic mater act as scavengers of the hydroxyl radicals and effect transformation products.	Michael et al. (2012)
Amoxicillin	Stimulated waste water and spiked STP effluent	Photo-Fenton	HPLC-DAD	- Maximum biodegradability and complete degradation were achieved at higher H_2O_2/COD molar ratios and which is depends on TOC content in WW effluents.	Elmolla and Chaudhuri (2009b) Trovo et al. (2008)
Triclosan	seawater and urban runoff wastewater	Sonochemical degradation	SPME/ GC–ECD	- Beneficial effect on degradation with increase in ionic strength (seawater > 3.5% NaCl > deionized water). - Concentration, composition and behavior of waste sludge has maximum effect (seawater > centrifuged urban runoff> untreated urban runoff > wastewater influent). - Physico-chemical properties of antibiotics effect technique efficiency.	Sanchez-Prado et al. (2008)
Sulfonamides Macrolide (erythromycin)	Spiked water and pharmaceutical wastewater	O_3 and O_3/H_2O_2	LC-MS	- Structural properties of antibiotics (C-C bond un saturation or saturation) effect efficiency and time of degradation. - Optimum pH depend on target compound and molar O_3/H_2O_2 ratio depends on water matrix type.	Lin et al (2009)
Tetracycline	Pure water, surface water and STP	Photo-Fenton	TOC, HPLC-UV	- Matrix effects the degraddation efficiency. - Under black-light matrix	Bautitz et al. (2007)

	effluent			lowers degradation and use of $FeNO_3$ nullifies matrix effects.	
Nirtoimidazoles	Ultrapure water surface water subterranean water and wastewater	Simultaneous application of ozonation and adsorption	HPLC-DAD, TOC	- Ozonation efficiency in WW was very less due to high dissolved TOC showed scavenging $OH^{\bullet}$ rate high compared to other matrix. - O_3/activated carbon: AC adsorb CO_3^- and HCO_3^- ions (decreased alkalinity) and also dissolved organic matter (20% reduction) results in improved efficiency.	Sánchez-Polo et al. (2008)
Fluoroquinolones	River and synthetic wastewater	Photocalatytic degradation	LC-MS/MS	- Slow degradation in river water was observed. - Chemical and structural properties have important role and structural cleavage and oxidative photo-degradation are mainly pH dependent.	Babić et al. (2013) Balcioğlu and Ötker (2003)
Enrofloxacin	Formulation and synthetic wastewater	Ozonation	BOD_5, COD and TOC	- Biodegradability was increased (BOD_5/COD). - More efficiently COD was removed with ozonation and its concentration is matrix dependent.	Guinea et al. (2009)

DW: demineralized water, SW: simulated natural freshwater, SWW: simulated wastewater, RE: real effluent, HPLC-UV-DAD: high performance liquid chromatography- ultra violet-diode array detector, UPLC-QToF/MS: ultra-pressure liquid chromatography-quadrupole time of flight/mass spectrometry, HPLC-DAD: high performance liquid chromatography-diode array detector, SPME/GC-ECD: solid-phase microextraction coupled with gas chromatography-electron capture detector, HPLC-UV: high performance liquid chromatography- ultra violet, LC-MS: liquid chromatography-mass spectrometry, LC-MS/MS: liquid chromatography-mass spectrometry/mass spectrometry.

Table 2.1.3 Comparison of individual and combined treatment technologies for wastewater comprising antibiotics

Matrix	Treatment technology	% Removal	Reference
Synthetic wastewater COD = 450 mg/l	O_3 2.96 g/1h O_3, pH 7 O_3/H_2O_2 2.96 g/1h O_3, pH 7, 20mM H_2O_2	- 69% COD and 29% UV_{254} removal - 100% of COD and UV_{254} removal	Balcioğlu and Ötker (2003)
Spiked surface water	Conventional membrane filtration Hybrid ozonation-membrane filtration ozone dosing 13.3 µg/s	< 40 % removal of organic matter > 99% removal of antibiotics > 80% TOC and UV_{254} reduction	Alpatova et al. (2013)
Industrial wastewater	Combined Fenton-SBR process with H_2O_2 52.9 mM Fe(II) 7.9 mM, and pH 4.0	leads to mineralization > 73% increase in BOD (0.13 to 0.5) process time is reduced (10 h to 4 h)	Wu et al. (2013)
WWTP effluents	Ozonation and BAC filtration	10 to 50% increase in DOC removal 90% reduction in trace organics and 40 to 70% reduction in toxicity	Reungoat et al. (2012)
Waste stream of tetracycline	Combined powdered ACs with ultrafiltration and reverse osmosis	> 95% removal of TOC, COD and tetracycline	Zhang et al. (2006)

BAC: biological activated carbon; COD, chemical oxygen demand; DOC: dissolved organic carbon; RO: reverse osmosis; SBR: sequential batch reactor; TOC, total organic carbon

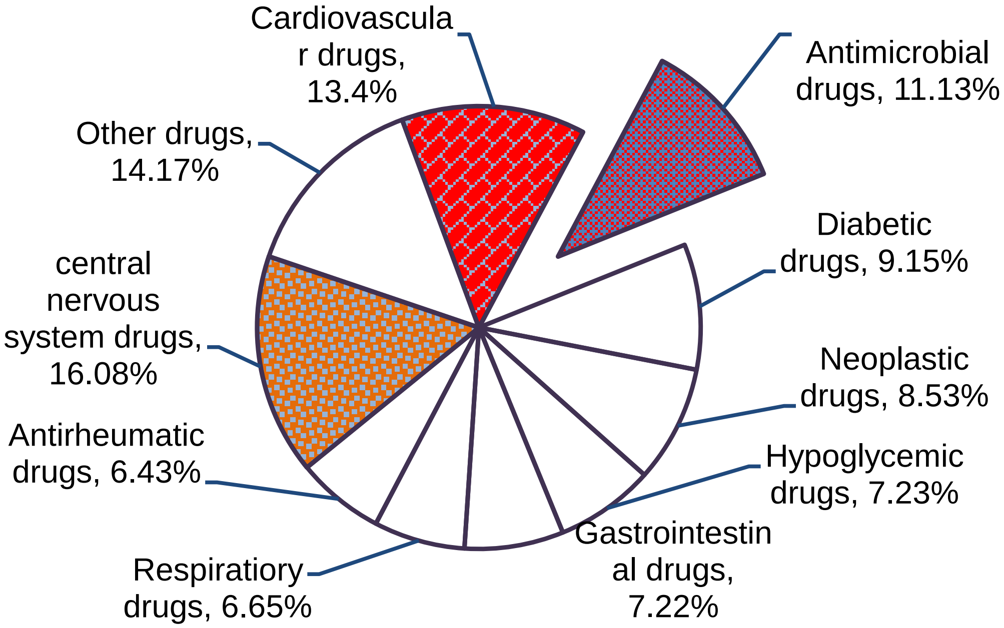


Figure 2.1.1 **Sales of different pharmaceutical drugs in world market**

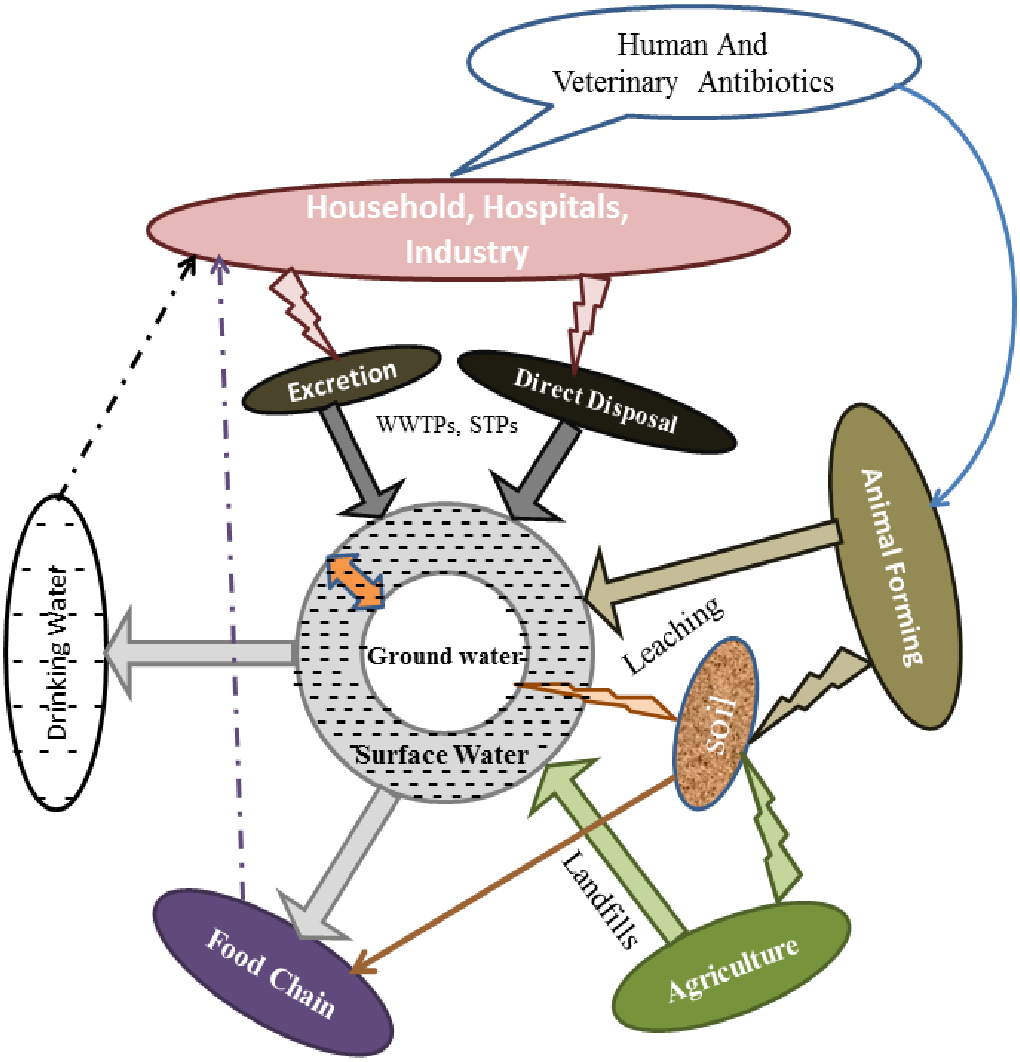
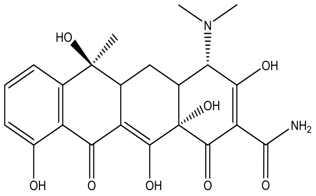
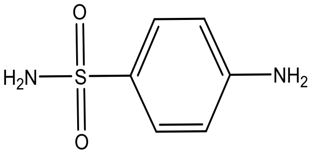
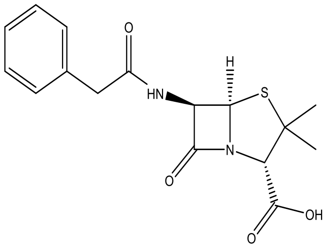
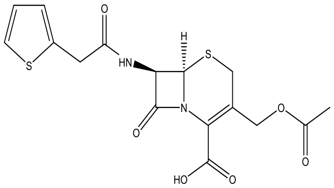
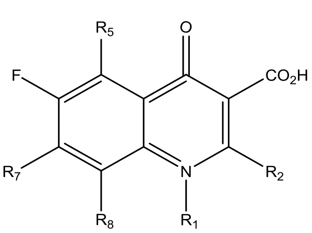


Figure 2.1.2 **Principal contamination routes of antibiotics and recycling**

Figure 2.1.3 Factors affecting the antibiotic degradation depending on treatment

Class	Core structure	Treatment	Deciding Fators	References
Tetracycline		Anodic oxidation (Pb/PbO_2) - activated sludge culture	- Increasing the temperature increased the degradation. - Initial higher antibiotic concentration has negative effect on degradation.	Idris et al. (2013) Kuixiao et al. (2008)
Sulfonamides		Photocatalytic process TiO_2 SBR-Ozonation	- Catalytic system composition. - pH values. - DOC strongly competed for oxidants.	Ewa et al. (2012) Ben et al. (2012)
β-lactams Penicillin		Photo-Fenton Sonolysis	- Initial Penicillin and H_2O_2 concentration. - pH of wastewater, COD and BOD.	Saghafinia et al. (2011)
Cephalosporin		Photolysis	- Presence of bicarbonate, nitrate and DOM stimulate photolysis.	Xiao-Huan et al. (2012
Quinilones		Sorption and biological degradation	- Suspended solids, temperature and the initial COD influence biodegradation kinetics. - Sorption depends upon quinolone type.	Dorival-García et al. (2013)

BOD, biological oxygen demand; COD, chemical oxygen demand; DOC: dissolved organic carbon; DOM: dissolved organic matter; TOC, total organic carbon

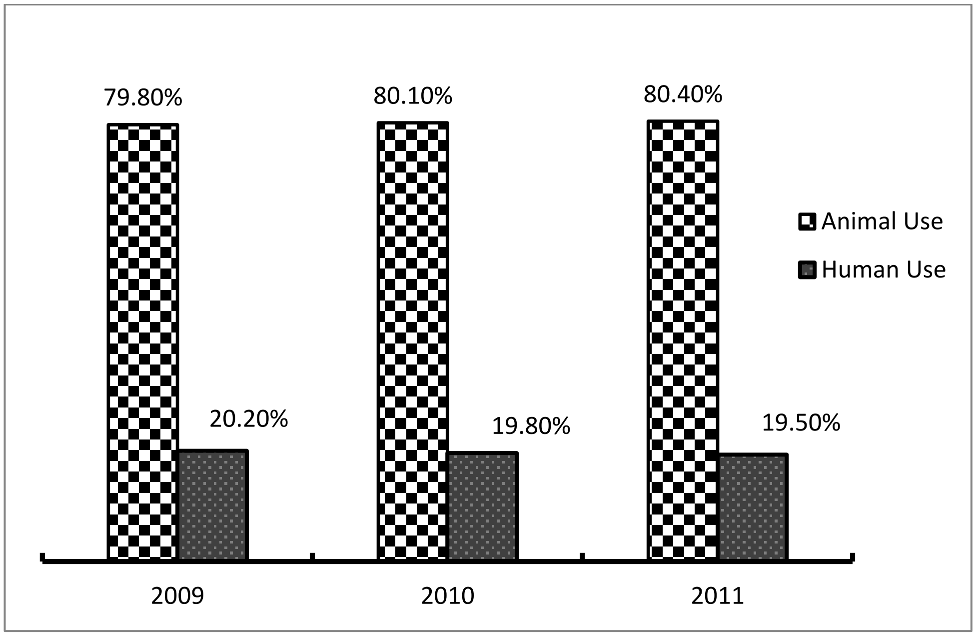
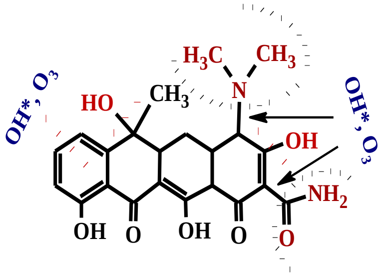
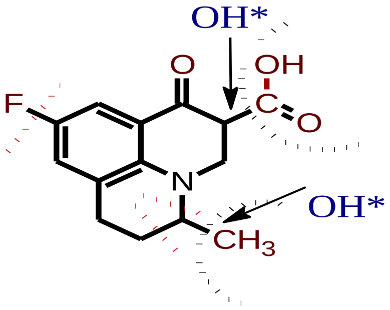
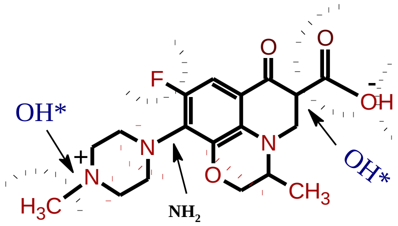
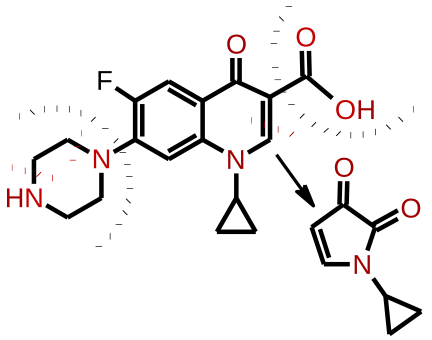
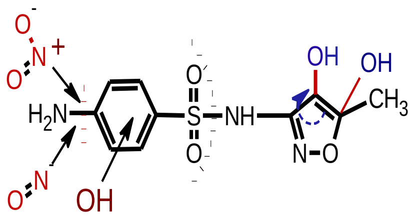
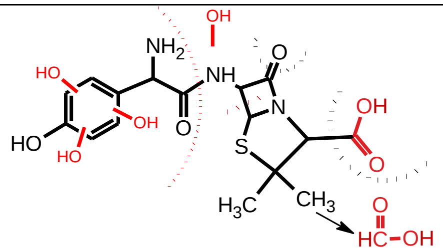


Figure 2.1.4 Consumption of antibiotics in human and livestock

Figure 2.1.5 Effect of physical-chemical parameters of compound and treatment method on degradation pathways

Antibiotic	Treatment	Conditions and detection	Degradation pathways and most liable groups	Physicochemical parameters (compound and method of treatment) controlling formation of byproducts	Results and comments	References
Tetracycline(TC)	Ozonation (O_3)	pH: 2.2 ±0.1, 7.0±0.1; O_3 - 10 mgmin^{-1}; TC - 0.5 mM. LCMS/MS		-- Presence of electron rich functional groups in target compound (double bonds, phenolic groups and amino group) is essential for ozonation. -- pH of the treatment method decides the direct (O_3) or indirect ($OH^{\cdot}$) reaction of TC.	-- Toxicity removed at both pHs (2.2 and 7.0) was similar (40%) but faster at pH 7.0, due to the non-selective reaction of free radicals with tetracycline and its ozonation byproducts at pH 7.0. -- 2 fold increased removal of total organic carbon at pH 7.0 compare to pH 2.2. -- Toxicity of effluent was varied due to different byproducts formed at different pHs. -- Wang et al. (2012) proved that combination of O_3 with ultrasonic irradiation increased the mass transfer rate of ozone and degradation of TC.	Khan, (2010) Yan et al. (2012)
Flumequine	Fenton and photo-Fenton	pH: 2.8, Flumequine - 500 mg L^{-1}; H_2O_2 - 0.5 to 10.0 mmol L^{-1}, Fe(II) - 0.25 to 1.0 mmol L^{-1}. HPLC, MS (ToF)		-- Occurrence of electronegative atom (fluorine (F)) undergo substitution and/or elimination reactions through $OH^{\cdot}$ or photo excitation. -- H_2O_2 concentration affect the efficiency of degradation, it must be optimized for the best.	-- In photo-Fenton process antimicrobial activity was reduced to 67% and in Fenton process it is reduced to 54% only, in 15 min. -- Here toxicity is related to degradation efficiency of flumequine, as formed byproducts loosed 3-carboxyl group which is necessary for biological activity.	Rodrigues-Silva et al. (2013)
Ofloxacin (OFX)	Sonophotocatalysis	H_2O_2 - 35% w/w solution, TiO_2 – 75:25 (rutile : anatase), OFX - 10 mg/L, light source UV - 350–400 nm. UPLC–MS/MS		-- Combined ultrasound and UV-A/TiO_2 process efficiency depended on many parameters such as ultrasonic power input, optimum H_2O_2 concentration and increased TiO_2 loading decreases the surface area for catalysis thus degradation . -- Catalysis is by hydroxide radicals; hence it is important that target compound must contain electron rich groups for oxidation reactions.	-- Increased toxicity of treated solution compared to untreated solution. -- Application of combined processes involves several mechanisms and pathways to degrade compound and limited by many parameters. -- Here the many formed transformation products (TPs) played a critical role with regard to the toxicity changes and limited the process efficiency and it is difficult to understand the oxidation pathways.	Calza et al. (2008) Hapeshi et al (2013)

Ciprofloxacin	Ozonation	Ciprofloxacin - 15 mg· L^{-1} O_3 - 2500 ppm_v HPLC/UV/HRMS		-- Existence of unsaturated binds and electron rich atom (nitrogen (N)) in target compound favors O_3 and $OH^{\cdot}$ attack. -- Formation of degradation products depends on the solution pH; as pH decides the generation of hydroxide radicals and position of attack in compound.	-- At different pHs (3, 7, 10) different TPs with different toxicity. -- At pH 3, TPs formed have antibacterial activity compared to TPs formed at pH 7 because at acidic pH direct ozone attack at piperazinyl ring and have less oxidation potential compared to hydroxide radicals for attacking quinolone ring, which is necessary for biological activity.	DeWitte et al. (2008) Liu et al. (2012)
Sulfamethoxazole (SMX)	Ozonation	SMX - 0.150 Mm, pH 2 and 8, H_2O_2/O_3 molar ratio < 0.5 LC-ESI-QTOF-MS		-- Based on the reactions with e^- rich atoms and double bonds, ozone was considered as a high selective electrophile at acidic pH. -- pH effects ozonation efficiency; in the absence of any pH control, during treatment solution pH depended on formed intermediates. -- Toxicity of solution was attributed to oxidation byproducts and depended on physical parameter time and ozone dosing; it is decreases with time and dose.	-- The dominance of intermediates formed one over the other depended on pH (2 and 8) and greater differences were observed in abundance. -- Direct ozonation is preferred for main degradation pathway of SMX (the cleavage of sulphonamide bond) at pH 2. -- Toxicological characterization of ozonized solution byproducts is necessary to avoid the release of toxic species in the environment.	Gómez-Ramos Mdel et al. (2011) Abellán et al. (2008)
Amoxicillin	Photo-Fenton process	AMX - 50 mg L^{-1}, $FeSO_4.7H_2O$ - 0.05 mM, Potassium ferrioxalate (K_3Fe $(C_2O_4)_3.3H_2O$) - FeOx - 0.05 mM, H_2O_2 - 120 mg L^{-1} LC/TOF-MS		-- Source of iron species for Fenton reaction effects the degradation time, efficiency and % of TOC removal. -- The benzoic ring and the nitrogen atom are susceptible to an electrophilic attack (OH*). -- Toxicity for both Fe species treatments due to carboxylic acids generated during degradation process	-- The hydroxylation and decarboxylation reactions yielded short chain carboxylic acids which allowing them for further conventional treatment. -- Significant toxicity during all the treatment was observed in case of ferrioxalate Fe species source due to the presence of oxalate in solution.	Trovó et al. (2011)

PART 2

A Persistent Antibiotic Partitioning and Co-relation with Metals in Wastewater Treatment Plant- Chlortetracycline

Rama Pulicharla[a], Dipti Prakash Mohapatra[a], Satinder Kaur Brar[a*], Patrick Drogui[a], Serge Auger[b], Rao.Y. Surampalli[c]

[a]INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

[b]Phytronix Technologies, 4535 boulevard Wilfrid Hamel, Québec, Canada, G1P 2J7

[c]Department of Civil Engineering, University of Nebraska-Lincoln, N104 SEC PO Box 886105, Lincoln, NE 68588-6105, US

(*Corresponding author, Telephone: + 418 654 3116; Fax: + 418 654 2600; Email: satinder.brar@ete.inrs.ca)

Journal of Environmental Chemical Engineering (2014) 2(6):1596-1603

Résumé

L'addition d'une forte concentration de l`antibiotique chlortétracycline (CTC) à l'alimentation du bétail et sa résistance à la dégradation entraînent une accumulation de CTC dans l'environnement. La forte activité chélatante du CTC avec les ions métalliques a des répercussions sur de nombreux facteurs tels que la solubilité, la stabilité, la dégradation et l'activité antibactérienne du CTC dans la station d'épuration des eaux usées. En outre, aucune étude n'a été menée sur le comportement de chélation de CTC avec des ions métalliques et son effet sur l'efficacité d'élimination de la station d'épuration. Cette étude tente de fournir des informations sur la distribution de CTC dans les eaux usées et les boues d'épuration et le rôle de la complexation des métaux CTC sur ce comportement. La méthode analytique développée dans cette étude combine une technique de prétraitement de l'extraction en phase solide (SPE) avec la désorption thermique par diode laser (LDTD) couplée à la spectrométrie de masse en tandem (MS/MS). L'analyse LDTD-MS/MS a été récemment développée pour améliorer la capacité de débit élevé dans la MS en réduisant les durées de LC-MS / MS de 5-30 min à 10-30 s dans la séquence LDTD-MS/MS. Cette étude a examiné les ions métalliques les plus courants, polluants et efficaces (Al(III), Ca(II), Co(II), Cu(II), Fe(III), Mg(II), Ni(II) et ZnII)) qui ont tendance à se complexer avec CTC en eaux usées. Le CTC-métal complexe et sa mobilité dans la station d'épuration étaient bien corrélés ce qui a montré que la concentration de CTC dans WWS était plus élevé que les eaux usées. Ces résultats ont montré que le CTC hydrosoluble est devenu insoluble et/ou relativement moins soluble après chélation avec des ions métalliques. De plus, les résultats indiquent l'importance de la propriété de chélation du CTC et servent d'outil pour déterminer la matrice de traitement des eaux usées à traiter, ainsi que le type de méthode de traitement à utiliser.

Mots clés: Chélation; Chlortétracycline; Ions métalliques; Eaux usées; Boues d'épuration.

Abstract

The addition of high concentration of chlortetracycline (CTC) antibiotic to livestock feed and its resistance towards degradation results in accumulation of CTC in the environment. The strong chelation activity of CTC with metal ions has impact on numerous factors, such as solubility, stability, degradation, and antibacterial activity of CTC in wastewater treatment plant (WWTP). Further, no studies have been carried out on the chelation behavior of CTC with metal ions and its effect on removal efficiency from WWTP. This study attempts to provide the information about CTC distribution in wastewater (WW) and wastewater sludge (WWS) and the role of CTC-metals complexation on this behavior. The analytical method developed in this study combines an existing pre-treatment technique of solid-phase extraction (SPE) with Laser Diode Thermal Desorption (LDTD) coupled with tandem mass spectrometry (MS/MS). LDTD-MS/MS analysis has been recently developed to enhance the high throughput capacity in MS by reducing LC-MS/MS runs of 5 to 30 min to 10 to 30 s in LDTD-MS/MS run. This study considered the most common, polluting and efficient metal ions (Al(III), Ca(II), Co(II), Cu(II), Fe(III), Mg(II), Ni(II) and Zn(II)) which have a tendency to complex with CTC in WW. Chlortetracycline-metal complex and its mobility in WWTP were well-correlated which showed that CTC concentration in WWS was higher than the WW. These results showed that the water soluble CTC became insoluble and/or relatively less soluble after chelation with metal ions. Furthermore, the results signify the importance of chelation property of CTC and serve as a tool in determining the wastewater treatment plant matrix to be treated and also the type of treatment method to be used.

Keywords: Chelation; Chlortetracycline; Metal ions, Wastewater; Wastewater sludge.

Introduction

Tetracyclines are old and broad-spectrum antibiotics; among tetracyclines, chlortetracycline (CTC) was the first member of the tetracycline family discovered in the late 1940s. The mechanism of action of these antibiotics to inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the bacterial ribosome is well established (Hierowski, 1965). The promising antibacterial properties of CTC and the absence of major adverse side effects has led to their common use in the treatment of human and animal infections. Apart from the treatment of infections, CTC is also used for prophylactic purposes; added at sub-therapeutic levels to animal feeds as growth promoters and to improve feed efficiency (Funk *et al.*, 2006). Currently, across the world, more than half of the antibiotics are used in the livestock, which contributes to the development of resistance. This has led to a ban of some antibiotics in humans, among them CTC is one (Prescott *et al.*, 2013). This antibiotic application at higher concentrations for therapeutic and prophylactic use and lower concentrations for growth promotion usually led to CTC, its degradation products and its epimers being discharged into the environment. Wastewater treatment plant (WWTP) effluent, biosolids and animal manure application to agricultural fields as fertilizer are regular sources of antibiotics into the environment (Kumar *et al.*, 2013, Romero *et al.*, 2013). Continuous usage of antibiotics for both infectious and non-infectious diseases resulted in global issues, such as antibacterial resistance in both human and veterinary medicine.

Heavy metals are essential in trace amounts for humans, animals and plants but large amounts of any of them may cause acute or chronic toxicity. Due to industrialization and urbanization, large amounts of heavy metal ions are released into the environment and become abundant (Hu *et al.*, 2013). Because of their high solubility in the aquatic environments, heavy metal ions can be absorbed and accumulated easily by living organisms and are toxic even at low concentrations. Metal ions in WW do not degrade naturally like organic pollutants. Toxicity of metal ions is due to direct generation of free radical species and depletion of antioxidants. The toxicity widely varies depending on the allotrope or oxidation state of the metal (Flora, 2009). Metal ions have inhibitory effects on the biological treatment process at the WWTPs and also limit the application of biosolids as fertilizer (Malamis *et al.*, 2012). Even though there are many treatment methods for removal of these toxic metal ions, but still, several metals are lacking robust pathways for effective removal from the WW. Apart from this, metal ions can get easily chelated with organic pollutants and remain in the WW at elevated pH and also change the chemical and clinical properties of chelated compounds (Valko *et al.*, 2005).

Chlortetracycline is the most regularly used pharmaceutical in intensive animal farming. Moreover, most of the studies so far have confirmed the presence of CTC in WW and WWS (Karthikeyan *et al.*, 2006, Kolpin *et al.*, 2002, Zilles *et al.*, 2005). Physico-chemical properties of CTC are shown in table 2.2.1. However, partitioning of CTC in the WWTP and the effect of presence of metal ions on partition has not been explored. Chlortetracyclines are strong chelating agents and their antimicrobial and pharmacokinetic properties are influenced by this chelation property. The presence of high concentrations of metal ions in WW is understood to account for the significant differences in distribution of CTC between WW and WWS. Further, the presence of metal ions in WWTP not only affect the partitioning of CTC but also have effect on other numerous factors such as stability, degradation, antibacterial activity. Chelation activity causes changes in the antibacterial properties leading to inferior antibacterial activity. This chelated metal-CTC complex undergoes pH-dependent speciation and therefore, might occur as charged species in the environment. Several studies have indicated positively charged metal-CTC complex traverse the outer membrane of gram-negative enteric bacteria leading to accumulation (Nikaido *et al.*, 1993). Uptake of metal-CTC complex across the cytoplasmic membrane is energy dependent and influenced by the pH of the surrounding environment (Schnappinger *et al.*, 1996).

Therefore, the present study was conducted with following objectives; (i) to develop a new, rapid and sensitive method for quantification of CTC in WW and WWS by using LDTD-MS/MS method; (ii) to evaluate the fate and partitioning of CTC in WWTP; (iii) to estimate different metal concentrations in the WW and WWS and; (iv) to find a correlation between CTC and metal ion concentration in WW and its critical link to distribution of CTC in WWTP.

Material and methods

Chemicals and standards

Isochlortetracycline (Iso-CTC, purity >97%) was purchased from Toronto research chemicals (TRC) (Toronto, Canada). Clomiphene citrate (purity >95%) salt was purchased from sigma Aldrich (USA). Nitric acid (HNO_3,Trace metal ™ grade, 67-70%), reagent grade perchloric acid ($HClO_4$, Trace metal ™ grade, 67-71%), hydrofluoric acid (HF, Trace metal ™ grade, 48-51%), methanol (HPLC grade, purity >99.8%), acetic acid (HPLC Grade, purity >99.7%), and ammonium hydroxide (NH_4OH, 99.5%, 28-30% w/w) were purchased from Fisher Scientific (Ontario, Canada). Disodium ethylenediamine tetraacetate (Na_2H_2EDTA, 99%) was purchased from E-bay (Tokyo, Japan). Standards for metal analysis were purchased from SCP Science, plasmaCAL, Quebec. Standard reference materials (SRM): peach leaves (SRM 1547) and Tart-3 (Lobster Hepato pancreas) were purchased from National Research Council of Canada, Ottawa, Canada. Sep-pack® C_{18} Plus Short Cartridge (360 mg Sorbent and 37-55 µm Particle Size per Cartridge) for solid phase extraction (SPE) were purchased from Waters (Milford, MA, USA). HPLC grade water was prepared in the laboratory using milli-Q/Millli-Ro Milli pore system (Milford, MA, USA)

Wastewater sampling and analysis

Samples were collected from Quebec city WWTP (Quebec, Canada) in October 2013. Samples were collected in pre-cleaned high-density polyethylene (HDPE) containers from six points of wastewater treatment plant, such as influent, grit, primary sludge, secondary sludge, mixed sludge (60% of primary sludge and 40% secondary sludge) and effluent on the same day. Samples were transported to laboratory and stored under dark conditions at $4 \pm 1\ ^0C$, until used. Wastewater control parameters, such as pH, total solids (TS), suspended solids (SS), volatile solids (VS), total chemical oxygen demand (TCOD), ammonia- nitrogen, phosphate, total organic carbon (TOC) and alkalinity were analyzed in accordance with the Standard Methods (APHA-AWWA-WPCF., 1981).

Sample preparation for metal analysis

General

Overall estimation of metal content in all liquid samples [influent, effluent and liquid fraction of sludge (primary sludge, secondary sludge and mixed sludge)] and solid samples (primary sludge, secondary sludge, mixed sludge and solid fraction of primary sludge, secondary sludge, and mixed sludge) was carried out by acid digestion using autoclave to get clear samples for ICP-AES metal analysis.

Liquid sample preparation

The liquid fraction was separated from sludge by centrifugation at 7650 *x* g for 25 min and the supernatant was hence transferred for acid digestion. About 4 mL of the supernatant was transferred to a Teflon test tube and 10 mL of concentrated HNO_3 was added for complete digestion. Autoclave digestion was performed in a pressure (17 psi) controlled manner. The digestion temperature was 120 ±1 ^{0}C and the digestion was performed for 2 h to get a clear solution for analysis. After digestion, the clear solution was transferred to a vial and made up to 20 mL with milliQ water.

Solid sample preparation

Solid fraction of sludge was separated by centrifugation at 7650 *x* g for 25 min. The resulted precipitate was dried in an oven at 105 ± 0.5 ^{0}C. The dried solid sample was weighed to about 0.2 g and transferred to a Teflon test tube. For complete digestion of solid sample, about concentrated 5mL of HNO_3, 100 µL of $HClO_4$ and 100 µL of HF were added. HF was further added to digest the silica present in the solid sample. After digestion, the clear liquid was transferred to a vial and made up to 50 mL with milliQ water. Reagent blank was prepared in the same acid composition and treated with the same digestion procedures as the samples but without the addition of sample itself.

A certified reference sample of Tart-3 and peach leaves was digested similar to WW samples for digestion method accuracy.

Standard preparation

Calibration standards were made using SCP Science provided PharmaCAL Standards. The standards were prepared in 10% $HNO_{3.}$ All standard and sample solutions were spiked with internal standard Yttrium (Y) 1 ppm. The spiking solution was taken from single element stock solution of Y 1000 ppm.

Sample preparation for CTC analysis

Wastewater sample preparation

Wastewater samples constituting dissolved and suspended particulate matter were centrifuged at 7650 *x* g for 25 min and the supernatant was filtered through an acetone washed 2 μm glass fibers (Fisher brand G6 filter circles, Fisher Scientific, Ontario, Canada). About 100 mL of filtrate was taken and the pH was adjusted with 2M acetic acid buffer to 3-3.5.

Solid sample preparation

Solid fraction of sludge samples were separated by centrifuging the WWS samples (primary sludge, secondary sludge and mixed sludge) at 7650 *x* g for 25 min. The resulting precipitate was frozen in refrigerator at -20 ^{o}C overnight before lyophilization by the freeze-dry system (Dura Frezz Dryer, Kinetics). The extraction of lyophilized samples was carried out by microwave assisted extraction method (MARS microwave extractor, CEM Corporation, North Carolina, USA). About 0.5 g of lyophilized sample was transferred to a Teflon test tube followed by addition of 20 mL of methanol. This procedure was repeated three times for every sample. This extraction allowed 14 vessels to be irradiated simultaneously. The extraction was performed in a pressure controlled mode at 120 psi and was programmed as follows: pressure ramp to 120 psi for 5 min and holding at 120 psi for 10 min. The extracts were combined, concentrated to volume of 1 mL with gentle stream of nitrogen and re-dissolved in 100 mL of milliQ water.

Clean-up

Sample extraction was conducted using Sep-pack® C_{18} Plus Short Cartridges. Position of cartridges were fixed in a vacuum manifold (Welch, USA) which was connected to a vacuum pump (Welch Rietschle Thomas, USA) for extracting the sample through cartridges. The SPE procedure was carried out as follows: 1) the pH of sample (WW and sludge) was adjusted to 3.5±0.5 with 2M acetic acid buffer and 0.08 g of

Na_2H_2EDTA was added to the sample to release the CTC from metal complexation; 2) the cartridges were preconditioned with 5 mL of methanol, 5 mL of deionized (DI) water and finally with 5 mL of deionized (DI) water having pH 3.5 ± 0.5 with a flow rate of approximately 1.5 mL/min; 3) the sample was loaded into the C_{18} cartridge and extracted at a flow rate of approximately 3 mL/min; after extracting the sample, the sorbents were dried using a vacuum system set at -15 psi for 30 min; (4) the CTC was eluted with 7 mL of methanol at a flow rate of 1.5 mL/min in order to give enough time of contact between solvent and adsorbed compounds; (5) the extract was dried under a gentle stream of N_2; and finally (6) the dried residue was reconstituted with 1 mL of a methanol/water mixture (75:25, v/v) before analysis. To determine the loss of compound during the SPE procedure CTC was spiked in 100 mL of WW sample (sample spiked) before extraction and for the matrix effects determination, CTC was spiked in 7 mL methanol extracts (matrix spiked) after extraction. The CTC concentrations were measured with the LDTD-MS/MS method developed in this study and spike recoveries were calculated.

LDTD-APCI-MS/MS analysis

Quantification of CTC concentration in WW and WWS samples were achieved by LDTD- APCI (atmospheric pressure chemical ionization) source (Phytronix technologies, Quebec, canada) mounted on a TSQ Ultra AM Mass Spectrometer (Thermo fisher scientific, Waltham, MA, USA). The LDTD-APCI source was equipped with a bar coded LazWell TM plate (96-well plate) to introduce liquid samples for analysis. Before spluttering the sample on the bottom of stainless steel-based LazWell; sample was mixed with EDTA (0.1 mg/mL) of solution for complete thermal desorption of CTC from plate. Samples were first spluttered (4 µL) onto the plate and left to dry at room temperature. A dried sample on the LazWell plate was then transferred to an *X-Y* movable stage of the LDTD housing unit. For thermal desorption of sample from plate, an infrared (IR) laser diode (980 nm, 20 W, continuous) was focalized on the back of the plate. The desorbed sample was carried through a glass tube by a stream of carrier gas (compressed air). The analyte in the sample was ionized at the end of the glass tube by APCI and analyzed by mass spectrometry. Quantification of CTC was determined by the internal standard method using clomiphene citrate (1 ng/L) as internal standard. A seven point calibration curve ranging from 5 ng/mL to 200 ng/mL was run by the use of standard composite solutions in a methanol/water mixture (75:25, v/v) and EDTA (0.1 mg/mL) solution. The area ratio was plotted against the analyte concentration ratio to obtain the calibration curve. Good linearity ($R^2 > 0.998$) of calibration curve was obtained.

The laser power pattern was optimized to control the temperature and the kinetics of thermal desorption which provides optimal signal for analysis. The laser power was ramped from 0 to 65% in 6 seconds, and maintained for 2 seconds. The APCI parameter settings were a positive corona discharge current of 3 µA, a carrier gas temperature of 30 ^{o}C and an air flow rate of 3 L/min. The API-5500 QTrap system was operated in multiple reactions monitoring (MRM) mode was used to quantify the analyte and clomiphene as internal standard (1ng/mL for all standards and samples) in a single experiment with a 200 ms dwell time. The analysis time was achieved in only 10 seconds from sample to sample with no traces of carry-over.

MS/MS optimized parameters in MRM with the highest intensity for the investigated CTCs and clomiphene citrate (IS) including their precursor and product ions, and collision energy are summarized in table 2.2.2. The product ion producing the highest intensity was used for MRM and quantitation to increase analytical sensitivity and selectivity.

Results and discussion

Method development for analysis of CTC in WW and WWS

Quantification studies

Accurate and rapid quantification of CTC was carried out using the LDTD-APCI source. Strong intensity peak was found in positive mode ionization for the analysis of CTC in WW and WWS. MS/MS scan was operated through MRM mode to quantify the analyte and the remaining parameters were optimized using the defined MRM scans. Specific 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. Maximum peak intensity was achieved with methanol/DI water (75:25, v/v) and for complete desorption of CTC from LazWell plate can be accomplished with the addition of EDTA. The relative standard deviation (RSD) was below 10% for all WW and WWS samples. The volume of the sample deposited on the LazWell was evaluated and it was found that 4 µL sample volume showed sharp peak as compared to broader peak with 8 µL..

In this work, the WW sample was adjusted to pH 3.5 ± 0.5 with 2M acetic acid buffer. It was reported that chlortetracycline antibiotics have remarkable stability problems when pH was below 2.5 and above 5 (Søeborg *et al.*, 2004). Heavy metal ions, such as Al(III), Ca(II), Co(II), Cu(II), Fe(III), Mg(II), Ni(II), and Zn(II) are commonly present in WW and can complex with CTC. Without the addition of EDTA to WW sample before extraction, there was only 50% recovery of CTC. The extraction efficiency was improved by releasing the CTC from CTC-metal complex by addition of EDTA which complexed with heavy metal ions. And also EDTA could have prevented the competitive binding of metal ions to the adsorbing sites of the C_{18} cartridge through EDTA-metal chelation**.**

Recovery and detection limits

The LDTD-APCI-MS/MS method with the optimized parameters was applied for quantification and method validation. SPE cartridges were selected for extraction of CTCs from WW matrices. Metal ions in WW may bind to silanol groups of these cartridges and reduce the active sites available for CTC adsorption; resulting in overall poor recovery. Apart from this, CTC binding potential with metal ions also lowered the recovery. EDTA was utilized in this study to chelate metals to prevent interference with the extraction of CTCs and also to trigger CTC release from metal complexes.

The recoveries of CTC from SPE cartridges were measured by extracting analytes from 100 mL of deionized water and all raw WWTP samples were spiked at 10 µg/L before and after extraction. Recoveries of all samples for CTCs were determined by applying the Equation 1.

$$\%\ \mathrm{recovery} = \left(\frac{\mathrm{C_m}}{\mathrm{C_o} + \mathrm{C_S}}\right) * 100 \qquad (\mathrm{Eq.\,1})$$

where, C_m=measured concentration of CTC in spiked sample, C_o=initial concentration of CTC observed in sample and C_s=concentration of CTC spiked in sample. Recoveries of CTCs spiked at 10 µg/L in deionized water were 95-105%, in WW samples the recoveries were 92-110% and in WWS and solid fraction of sludge samples recoveries were 80-90%. Recoveries of CTCs in the deionized water and WW samples were similar indicating that matrix effects were minimal. Lower recoveries of WWS samples compared to WW samples indicated the presence of complex mixture of various organic and inorganic substances in sludge matrices. Solubility of these substances in the given extraction and eluent solvents may affect the recovery of CTC. Previous studies have reported that matrix effects resulted in suppression or enhancement of the analyte signal; and it mainly depends on the ionization source (Mallet *et al.*, 2004). Tetracyclines were also able to form stable ternary complexes (MacKay *et al.*, 2005); this may affect the extraction efficiency and recovery of compound. These results indicated that SPE cartridges gave reproducible recoveries for CTCs; which demonstrated the validity of the proposed method for the isolation of the CTCs.

The calibration curve with seven concentration levels (5-200 ng/L) for quantification of CTC in WW and WWS showed good linearity with correlation coefficient (R^2) of 0.998 and 0.997 respectively. Limits of detection (LOD) based on a signal-to-noise (S/N) ratio of 3 was measured using the standard error of the intercept and the slope of the calibration curve. The precision, intra-day repeatability and inter-day reproducibility were expressed as relative standard deviation (RSD), these results are summarized in table 2.2.3.

Distribution of CTC in WWTP

The basic characteristics of wastewater collected in Quebec Urban community WWTP are summarized in table 2.2.4. The average wastewater stream flow during the sampling periods was 2.14×10^4 m^3/d. The annual average dewatered sludge production was about 57.5 tons/d.

The detailed information on occurrence of CTC concentrations in WW and WWS samples collected from different treatment units in the WWTP are reported in table

2.2.5. Chlortetracycline was detected at the levels above their LOD in all wastewater samples with concentration as high as 61.46 µg/L in raw influent and 7.28 µg/L in effluent. Such a high concentration detected in wastewater was probably due to common use of CTC in sub-therapeutic and therapeutic levels to treat broad veterinary infections in Quebec. High concentration of CTC in WWTP was also reported in WWTPs in other countries, such as 32.67 µg/L in China (Ben *et al.*, 2008) and 0.69 µg/L in U.S. Previous studies reported that CTC was not detected in the WWTP effluents of Canada (Miao *et al.*, 2004). However, this study showed higher concentration of CTC in influent which indicated the correlation with increased feeding of CTC in livestock for growth promotion and improved feed efficiency in Canada in 10 years. It was observed that treatment process appeared to effectively remove this compound from WW, reducing their concentrations by 90% in the final effluent.

In the present study, higher concentration of CTC was observed in the WWS as compared to WW. All measured metal ion concentrations in sludge samples decreased in following order: primary sludge>secondary sludge>mixed sludge (except aluminum). Further, CTC concentrations of 804.87 µg/kg, 638.89 µg/kg and 469.09 µg/kg, respectively were observed in WWS showing the trend in decreasing concentration of CTC along the treatment chain (primary sludge>secondary sludge>mixed sludge) in relation to metal concentrations. TOC concentration in primary and secondary sludge was observed to be 741.63 g/L and 411.82 g/L, respectively. High concentrations of TOC in sludge (sorption) also contribute to the retention of CTC in WWS (Gu *et al.*, 2008). As CTC has a solubility of 8.6 mg/mL in water hence expected to retain in higher concentration in WW when compared to WWS. However, the chelation property of CTC with metal ions made this compound insoluble or less soluble in water and sorption to sludge phase led to retention in the sludge. Christian *et al.* (Christian *et al.*, 2003) and Gu *et al*., 2008 studied the effect of sludge retention time, pH and metal concentrations on the sorption of tetracyclines to organic matter. They observed the formation of ternary complex (humic matter-metal-tetracycline) at different metal concentrations and variable pH. CTC sorption to sludge phase played an important role in preventing the entry of the compound in effluent. And moreover, observed higher concentrations of CTC in soil indicated the application of biosolids to agricultural field and landfill leading to accumulation of this compound in the soil and further recycling via food chain (Aust *et al.*, 2008, Toth *et al.*, 2011).

The mass flow and distributions in WW and WWS were estimated to evaluate the removal process in the WWTP. The average mass flow of CTC was calculated (Equation 2) as the measured concentrations from the wastewater samples multiplied by the average waste stream flow rates along with each wastewater treatment step.

$$m = Q \times S \qquad \text{Eq. 2}$$

where, m = mass flow (g/L), Q = stream flow (m^3/d or tons/d) and S = Concentration of CTC (µg/L or µg/kg). Mass balance proved the distribution of CTC in WW and WWS, as well as the removal efficiency of WWTP. In this study, the treatment plant was capable of removing nearly 90% of CTC. This was mainly due to the contribution of metal complexation and sorption process to organic matter in the sludge and the biological degradation seemed to be insignificant. Figure 2.2.1 shows the mass balance of CTC along the treatment plant chain.

Metals concentrations in WWTP

Increased attention towards industrialization and its expansion mainly results in release of toxic metal ions into the environment. In this study, only selected metals were analyzed which had a tendency to form complex with CTC and were toxic. Metals concentrations in WW samples were determined using ICP-AES. The resolution of the system was sufficiently good for typical analysis of environmental samples. Instrument detection limits (IDLs) were estimated by taking 10 replicate measurements of the calibration blank and then multiplying the standard deviation (SD) by a factor of 3. Selected wavelengths and IDL are summarized in table 2.2.6. The results of analysis of metal concentrations in CUQ WWTP at all sampling points is presented in table 2.2.7. The validity of the calibration was checked by analyzing the Quality Control standards at selected intervals to ensure that the instrument performance remained consistent over the length of the analysis. Table 2.2.8 provides the QC recoveries in % at the beginning and end of the analysis for validating the instrument performance. The developed method was further validated by spike recovery studies and the recoveries were well within the limits of 92 to 110%. The accuracy of the metal analysis in WW samples were calculated as the difference between measured and certified concentrations for the CRM. Results are presented in tables 2.2.9 and 2.2.10. Precision (%RSD) was also measured to ensure the short term stability of emission signals for all elements and samples and it was below 5% for all. Al, Co, Ni, and Zn metal ion concentrations in wastewater influent and effluent were below detection limits (BDL). However, in sludge samples, Al, Co, Ni, and Zn metal ions were detected to be 70 to 355 mg/L, 0.15 to 0.35 mg/L, 0.14 to 0.47 mg/L and 3.4 to 14 mg/L respectively.

CTC-metal complexation in WWTP

High concentrations of metal ions in WWS (nearly 10 to 100 times) and corresponding higher concentrations of CTC in sludge compared to WW samples represented a correlation between metal and CTC concentrations. The investigated CTC was detected as high concentrations in collected sludge samples compared to wastewater. CTC is an organic acid with a pKa 3.3 and hence exhibits predominantly as anionic species at pH 6-7. Apart from this, the chelation activity of CTC with metal ions present in wastewater form CTC-metal complex and become insoluble or less soluble. The main chelation sites include the β-diketone system (positions 11 and 12) and the enol (positions 1 and 3) and carboxamide (position 2) groups of the A ring as shown in Figure 2.2.2 (Chopra *et al.*, 2001). Because of pH dependent speciation of CTC, many mechanisms are involved in the interaction of CTC with sludge, such as: (i) negatively charge phenolic diketone moiety of CTC may interact with metal ions via cation bridging; (ii) positively charged quaternary ammonium functional group of CTC may interact with negatively charged sorption sites on the sludge (humic and fulvic acid); and (iii) strong sorption to WWS via complexation with metal ions associated with sludge and cation exchange reaction.

This relationship was mainly due to the chelation property of CTC with metal ions. Presence of more hydroxide groups and carboxamide thermodynamically assisted CTC to bind with metal ions at different positions. Not only has the metal ion complexation, sorption with and without metal ion complexation of CTC to organic matter made this compound to be retained in sludge, despite its hydrophilicity. Recently, many studies have reported environmentally detected concentrations (2-30 µg/Kg) in soil and plants compared to surface water reflecting the application of activated sludge with accumulated CTC as fertilizer leads to bio magnification in soil (Aust *et al.*, 2008, Hawker *et al.*, 2013, Toth *et al.*, 2011).

Conclusions

A new rapid and sensitive method for quantification of chlortetracycline in wastewater sample has been developed using solid-phase extraction and LDTD-MS/MS. A simple and efficient SPE procedure was developed for clean-up and enrichment of the CTC in the effluent. The method was validated by studying linearity, the recovery, precision and the MDL and the method was applied to determine concentrations of CTC in different samples of WW. This study demonstrated that CTC was present in WW samples in varying concentrations ranging from 61 µg/L to 8 µg/L and in sludge samples ranging from 800 µg/kg to 400 µg/kg.

In spite of hydrophilic nature of the CTC, high concentrations were measured in sludge sample and not in WW. This indicated that chelation behavior of CTC with metal ions and sorption of these CTC-metal complexes to organic matter resulted in accumulation of CTC in sludge phase. Higher concentration of CTC was measured in primary sludge which was about 804 µg/kg and contained higher total solids (17.76 g/L) and higher metals concentration (Al-354.54 mg/L, Ca-710.29 mg/L, Fe-469.07 mg/L, Mg-86.60 mg/L, Zn-14.04 mg/L) compared to secondary and mixed sludge samples. These results have demonstrated the fact that application of wastewater sludge as fertilizer is an important pollution source of CTC in soil. Nevertheless, the measured effluent concentration (7.8 µg/L) was further diluted when released into the ecosystem; yet continuous use and persistent nature (half-life) of CTC might lead to bioaccumulation. Overall, the results from this study helped in understanding the transport and distribution of CTC in WWTP, and further in developing control and treatment measures to degrade CTC in WWS sample and to prevent the release of antibiotics to the environment. Therefore, it is important to measure not only the removal efficiency of pharmaceuticals in WWTPs, but also the quantities that are retained in biosolids and especially the liquid-solid partitioning.

Acknowledgement

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Discovery Grant 355254), and Ministère des Relations internationales du Québec (coopération Québec-Catalanya 2012-2014) for financial support. The views or opinions expressed in this article are those of the authors.

References

APHA-AWWA-WPCF. (1981) *Standard methods for the examination of water and wastewater.* APHA American Public Health Association,

Aust M-O, Godlinski F, Travis GR, Hao X, McAllister TA, Leinweber P & Thiele-Bruhn S (2008) Distribution of sulfamethazine, chlortetracycline and tylosin in manure and soil of Canadian feedlots after subtherapeutic use in cattle. *Environmental Pollution* 156(3):1243-1251.

Ben W, Qiang Z, Adams C, Zhang H & Chen L (2008) Simultaneous determination of sulfonamides, tetracyclines and tiamulin in swine wastewater by solid-phase extraction and liquid chromatography–mass spectrometry. *Journal of Chromatography a* 1202(2):173-180.

Chopra I & Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and molecular biology reviews* 65(2):232-260.

Christian T, Schneider RJ, Färber HA, Skutlarek D, Meyer MT & Goldbach HE (2003) Determination of antibiotic residues in manure, soil, and surface waters. *CLEAN–Soil, Air, Water* 31(1):36-44.

Flora SJ (2009) Structural, chemical and biological aspects of antioxidants for strategies against metal and metalloid exposure. *Oxidative medicine and cellular longevity* 2(4):191-206.

Funk JA, Lejeune JT, Wittum TE & Rajala-Schultz PJ (2006) The effect of subtherapeutic chlortetracycline on antimicrobial resistance in the fecal flora of swine. *Microbial drug resistance* 12(3):210-218.

Gu C & Karthikeyan K (2008) Sorption of the antibiotic tetracycline to humic-mineral complexes. *Journal of environmental quality* 37(2):704-711.

Hawker DW, Cropp R & Boonsaner M (2013) Uptake of zwitterionic antibiotics by rice (Oryza sativa L.) in contaminated soil. *Journal of hazardous materials* 263:458-466.

Hierowski M (1965) Inhibition of protein synthesis by chlortetracycline in the E. coli in vitro system. *Proceedings of the National Academy of Sciences* 53(3):594-599.

Hu Y, Liu X, Bai J, Shih K, Zeng EY & Cheng H (2013) Assessing heavy metal pollution in the surface soils of a region that had undergone three decades of intense industrialization and urbanization. *Environmental Science and Pollution Research* 20(9):6150-6159.

Karthikeyan K & Meyer MT (2006) Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Science of the Total Environment* 361(1):196-207.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB & Buxton HT (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999− 2000: A national reconnaissance. *Environmental science & technology* 36(6):1202-1211.

Kumar RR, Park BJ & Cho JY (2013) Application and environmental risks of livestock manure. *Journal of the Korean Society for Applied Biological Chemistry* 56(5):497-503.

MacKay AA & Canterbury B (2005) Oxytetracycline sorption to organic matter by metal-bridging. *Journal of environmental quality* 34(6):1964-1971.

Malamis S, Katsou E, Daskalakis N & Haralambous KJ (2012) Investigation of the inhibitory effects of heavy metals on heterotrophic biomass activity and their mitigation through the use of natural minerals. *Journal of Environmental Science and Health, Part A* 47(13):1992-1999.

Mallet CR, Lu Z & Mazzeo JR (2004) A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. *Rapid Communications in Mass Spectrometry* 18(1):49-58.

Miao X-S, Bishay F, Chen M & Metcalfe CD (2004) Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. *Environmental science & technology* 38(13):3533-3541.

Nikaido H & Thanassi D (1993) Penetration of lipophilic agents with multiple protonation sites into bacterial cells: tetracyclines and fluoroquinolones as examples. *Antimicrobial agents and chemotherapy* 37(7):1393.

Prescott JF & Dowling PM (2013) *Antimicrobial therapy in veterinary medicine.* John Wiley & Sons,

Romero C, Ramos P, Costa C & Márquez MC (2013) Raw and digested municipal waste compost leachate as potential fertilizer: comparison with a commercial fertilizer. *Journal of Cleaner Production* 59:73-78.

Schnappinger D & Hillen W (1996) Tetracyclines: antibiotic action, uptake, and resistance mechanisms. *Archives of microbiology* 165(6):359-369.

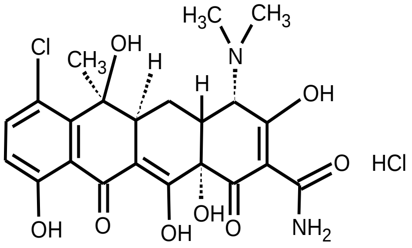
Søeborg T, Ingerslev F & Halling-Sørensen B (2004) Chemical stability of chlortetracycline and chlortetracycline degradation products and epimers in soil interstitial water. *Chemosphere* 57(10):1515-1524.

Toth JD, Feng Y & Dou Z (2011) Veterinary antibiotics at environmentally relevant concentrations inhibit soil iron reduction and nitrification. *Soil Biology and Biochemistry* 43(12):2470-2472.

Valko M, Morris H & Cronin M (2005) Metals, toxicity and oxidative stress. *Current medicinal chemistry* 12(10):1161-1208.

Zilles J, Shimada T, Jindal A, Robert M & Raskin L (2005) Presence of macrolide-lincosamide-streptogramin B and tetracycline antimicrobials in swine waste treatment processes and amended soil. *Water environment research* 77(1):57-62.

Table 2.2.1 Physico-chemical properties of Chlortetracycline hydrochloride

Structure	
Molecular formula	$C_{22}H_{23}ClN_2O_8$ HCl
Molecular weight	515.34 Da
Density	1.7 ± 0.1 g/cm^3 at 25 °C
Water solubility	8.6 mg/mL at 25 °C
Melting point	210–215 °C
Boiling point	821.1 ± 65.0 °C at 760 mmHg
Vapor pressure	0.0 ± 3.1 mmHg at 25 °C
log $K_{o/w}$	-0.53 ± 0.82
pKa*	3.33 ± 0.30 7.55 ± 0.02 9.33 ± 0.30
Storage	-20 °C
Appearance	The yellow crystalline solid
Use	An antimicrobial and antibacterial agent

*pKa values were measured by potentiometric method [27]

Table 2.2.2 Precursor and product ions and collision energy (CE) for the investigated pharmaceutical compound, chlortetracycline (CTC)

	Precursor ion Mass (Da), [M + H]+, (m/z)	**Product ion Mass (Da)**	**Collision energy (CE) (volts)**
CTC	**479**	**211**	**45.00**
	479	444	40.00
	479	462	40.00
Clomiphene	**406**	**100**	**35.00**

Table 2.2.3 Chlortetracycline method validation parameters

	R^2	**Intra-day precision (%RSD)**	**Inter-day precision (%RSD)**	**IDL (µg/L)**	**MDL (µg/L)**	
					LOD	**LOQ**
Wastewater	0.998	4.22	7.53	1.67	5.13	17.54
Wastewater sludge	0.997	9.18	12.39	2.49	7.49	25.92

RSD: Relative Standard Deviation; IDL: Instrument Detection Limit; MDL: Method Detection Limit; LOD: Limit of Detection; LOQ: Limit of Quantification

Table 2.2.4 Wastewater characterization parameters of Quebec Urban Community (QUC) wastewater treatment plant (WWTP)

Parameters	Influent	Screened sample	Primary sludge	Secondary sludge	Mixed sludge	Secondary sludge with chemical added	Effluent
pH	7.58±0.23	7.11±0.36	6.82±0.33	6.14±0.41	5.85±0.12	5.89±0.2	7.46±0.3
TS (g/L)	1.43±0.02	0.93±0.05	17.76±0.15	9.15±0.13	13.33±0.24	8.42±0.19	0.54±0.01
SS (g/L)	0.74±0.001	0.43±0.04	14.92±0.5	7.22±0.35	7.91±0.21	6.81±0.15	0.28±0.08
VS (g/L)	0.11±0.05	0.27±0.03	10.01±0.25	5.02±0.42	6.30±0.41	5.43±0.33	0.02±0.003
TCOD (g/L)	0.69±0.03	5.49±1.0	9.6±0.7	6.11±1.4	7.87±0.14	5.87±0.22	0.36±0.05
Ammonical nitrogen (g/L)	0.022±0.01	0.016±0.03	0.13±0.02	0.21±0.01	0.27±0.03	0.24±0.04	0.003±0.0001
Phosphorus (g/L)	0.0026±0.001	0.003±0.001	0.16±0.02	0.35±0.01	0.42±0.02	0.32±0.02	0.0013±0.0001
TOC (g/L)	0.0623±0.001	43.93±0.43	741.63±0.57	411.82±0.66	559.23±0.79	392.85±0.49	0.04±0.01
Alkalinity (g/L)	0.2065±0.05	0.21±0.03	0.965±0.16	1.14±0.29	1.245±0.12	1.095±0.17	0.135±0.05

Table 2.2.5 Concentrations of chlortetracycline in wastewater and wastewater sludge samples collected from Quebec Urban Community (QUC) wastewater treatment plant (WWTP) (n = 3).

	Concentration ±SD (n=3) (µg/L)	**Recovery Efficiency (%)**		**Concentration ±SD (n=3) (µg/g)**	**Recovery Efficiency (%)**
Influent	61.46±0.36	94.23	Primary sludge	0.804±0.03	89.32
Screened Sample	57.63±0.29	97.56	Secondary sludge	0.638±0.08	90.42
Primary sludge liquid	20.67±0.22	108.09	Mixed sludge	0.469±0.05	88.32
Secondary sludge liquid	31.22±0.36	96.74	Primary sludge solid	0.739±0.07	82.73
Mixed sludge liquid	35.74±1.49	106.10	Secondary sludge solid	0.615±0.02	86.23
Effluent	7.28±0.171	92.81	Mixed sludge solid	0.431±0.04	87.71

Table 2.2.6 List of wavelengths selected and detection limits

Analyte	**Wavelength (nm)***	**Correlation coefficient**	**Instrument detection limits (mg/L)**	**Method detection limits (mg/L)**
Al	308.215	0.9997	0.00084	0.0028
Ca	315.887	09999	0.00173	0.0058
Co	228.615	0.9999	0.00078	0.0026
Cu	324.754	0.9998	0.00091	0.0030
Fe	261.187	0.9999	0.00381	0.0127
Mg	280.270	0.9999	0.00024	0.0008
Ni	231.604	0.9989	0.00068	0.0023
Zn	213.857	0.9999	0.00024	0.0008

*wavelengths are selected based on minimum interference not based on maximum intensity.

Table 2.2.7 Sample analysis results by ICP-AES

Element	**Influent (mg/L)**	**Screened sample (mg/L)**	**Primary sludge (mg/Kg)**	**Secondary sludge (mg/Kg)**	**Mixed sludge (mg/Kg)**	**Effluent (mg/L)**
Al	BDL	BDL	19963.01	7723.60	20704.84	BDL
Ca	51.58	37.6	37577.27	11863.76	11265.28	46.44
Co	BDL	BDL	19.72	20.17	10.96	BDL
Cu	0.018	0.0055	317.78	359.40	215.46	0.009
Fe	1.53	0.121	26351.88	14697.09	10256.78	0.183
Mg	7.78	7.90	4404.72	2396.86	2130.63	6.97
Ni	BDL	BDL	26.65	14.40	10.44	BDL
Zn	BDL	BDL	790.63	370.07	324.42	BDL

*BDL= Below Detection limit

Table 2.2.8 Quality Control (QC) check standard recoveries

Element	**QC standards % recovery**	
	Beginning	**End**
Al	96.73±0.5	91.22±0.5
Ca	99.03±0.8	98.43±0.9
Co	99.30±1.5	99.4±1.4
Cu	99.42±0.9	95.73±0.6
Fe	98.92±0.8	97.21±0.9
Mg	98.99±0.8	98.65±0.5
Ni	99.56±0.9	93.81±0.3
Zn	100.80±0.9	101.49±1.2

Table 2.2.9 Analysis of Tart-3 SRM recovery - all values are in mg/kg; dashed lines indicate no certified value was provided by the manufacturer

Analyte	Wavelength (nm)	Certified values (mg/Kg)	Found Values (mg/kg)	Recovery (%)
Al	308.215	-----	-------	--------
Ca	315.887	------	--------	-----------
Co	228.615	1.06*	0.97	91.35±5.5
Cu	324.754	-----------	-------	-------
Fe	261.187	179±8	160.62	101.37±0.5
Mg	280.270	--------	--------	-------
Ni	231.604	5.30±0.24	4.75	89.58±0.8
Zn	213.857	136±6.0	131.26	96.52±0.6

Table 2.2.10 Analysis of peach leaves SRM recovery - all values are in mg/kg; dashed lines indicate no certified value was provided by the manufacturer

Analyte	Wavelength (nm)	Certified values (mg/Kg)	Found Values (mg/kg)	Recovery (%)
Al	308.215	249±8	240.28	96.50±1.0
Ca	315.887	15600±0.02	15315.66	98.18±0.4
Co	228.615	---------	----------	------
Cu	324.754	-----------	-------	-------
Fe	261.187	-------------	--------	----------
Mg	280.270	4320±0.008	4252.53	98.44±0.4
Ni	231.604	0.69±0.09	0.71	102.47±2.6
Zn	213.857	17.9±0.4	17.32	96.78±6.0

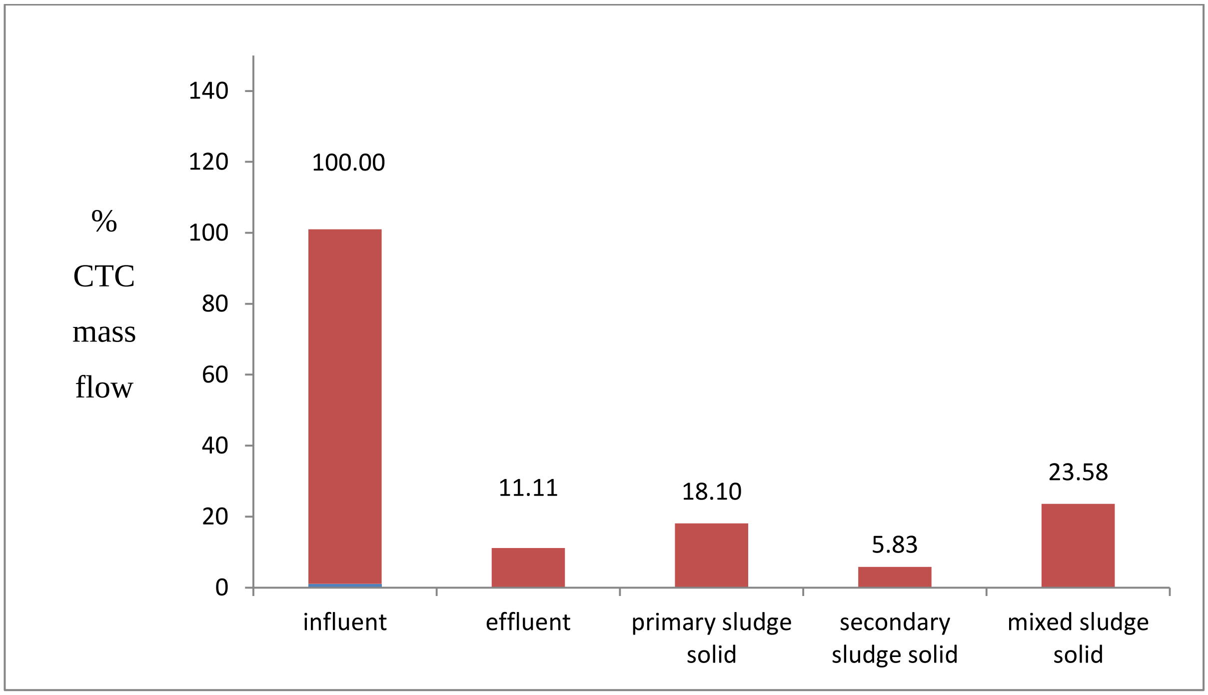


Figure 2.2.1 Percentage of detected CTC along with the treatment plant

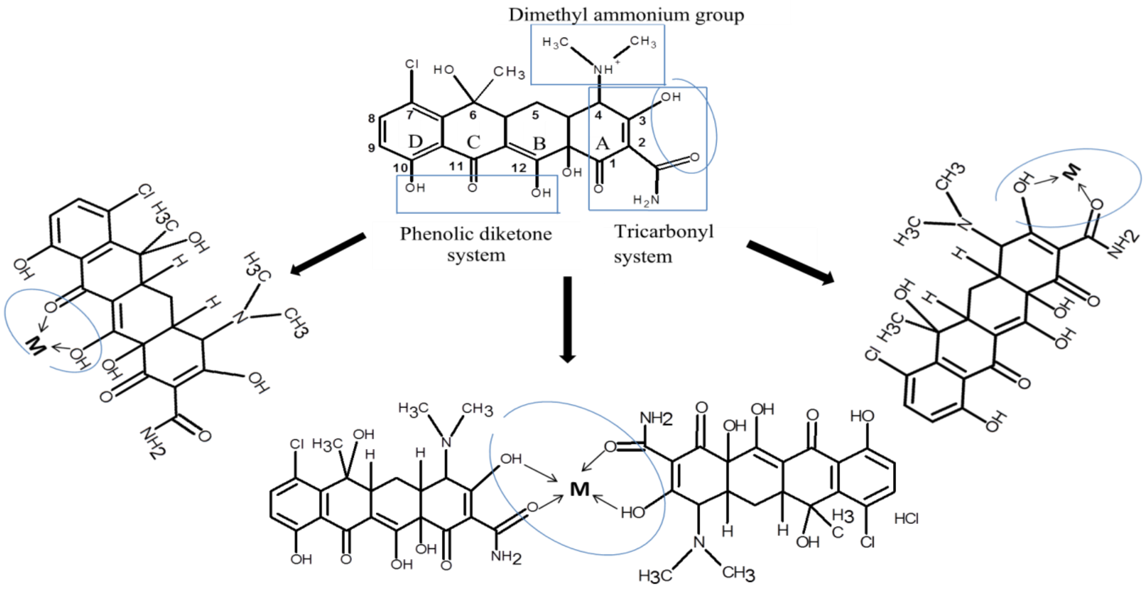


Figure 2.2.2 Chlortetracycline (CTC) complexation with metal ions at different positions

CHAPTER 3

TOXICITY OF CTC AND ITS METAL COMPLEXES

PART 1

TOXICITY OF CHLORTETRACYCLINE AND ITS METAL COMPLEXES TO MODEL MICROORGANISMS IN WASTEWATER SLUDGE

Rama Pulicharla[a], Ratul Kumar Das[a], Satinder Kaur Brar[a]*, Patrick Drogui[a], Saurabh Jyoti Sarma[a], Mausam Verma[b], Rao Y. Surampalli[c], Jose R. Valero[a]

[a] INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9.
[b]CO2 Solutions Inc., 2300, Rue Jean-Perrin, Québec, Québec G2C 1T9 Canada.
[c] Department of Civil Engineering, University of Nebraska-Lincoln, N104 SEC PO Box 886105, Lincoln, NE 68588-6105, USA.

(*Corresponding author, Telephone: + 418 654 3116; Fax: + 418 654 2600; Email: satinder.brar@ete.inrs.ca)

Science of The Total Environment (2015) 532:669-675

Résumé

La complexation des antibiotiques avec les métaux est un phénomène bien connu. Les usines de traitement des eaux usées contiennent des métaux et des antibiotiques, il est donc essentiel de connaître l'effet de ces complexes sur la toxicité vis-à-vis des microorganismes, typiquement présents dans les procédés de traitement secondaire. Dans cette étude, les constantes de stabilité et la toxicité des complexes chlortétracycline (CTC) et métal (Ca, Mg, Cu et Cr) ont été étudiées. Les constantes de stabilité calculées des complexes CTC-métal suivaient l'ordre: Mg-CTC> Ca-CTC> Cu-CTC> Cr-CTC. Les bacteries *Bacillus thuringiensis* (Bt), Gram positive, et *Enterobacter aerogenes* (Ea) Gram negative, ont été utilisées comme microorganismes modèles pour évaluer la toxicité de la CTC et de ses complexes métalliques. Les complexes CTC-métal étaient plus toxiques que le CTC lui-même pour Bt alors que pour Ea, CTC et ses complexes métalliques présentaient une toxicité similaire. En revanche, les boues de traitement des eaux usées de CTC n'ont montré aucun effet toxique par rapport aux eaux usées synthétiques. Cette étude fournit des preuves que la CTC et ses complexes métalliques sont toxiques pour les bactéries lorsqu'elles sont biologiquement disponibles. En ce qui concerne WWS, le CTC a été adsorbé à une partie solide et n'était pas disponible biologiquement pour montrer des effets toxiques mesurables.

Mots clés: Bacterie; Chlortétracycline; Complexation métallique; Toxicité; Boue d'eaux usées.

Abstract

Complexation of antibiotics with metals is a well-known phenomenon. Wastewater treatment plants contain metals and antibiotics, thus it is essential to know the effect of these complexes on toxicity towards microorganisms, typically present in secondary treatment processes. In this study, stability constants and toxicity of chlortetracycline (CTC) and metal (Ca, Mg, Cu and Cr) complexes were investigated. The calculated stability constants of CTC-metal complexes followed the order: Mg-CTC>Ca-CTC>Cu-CTC>Cr-CTC. Gram positive *Bacillus thuringiensis* (Bt) and Gram negative *Enterobacter aerogenes* (Ea) bacteria were used as model microorganisms to evaluate the toxicity of CTC and its metal complexes. CTC-metal complexes were more toxic than the CTC itself for Bt whereas for Ea, CTC and its metal complexes showed similar toxicity. In contrast, CTC spiked wastewater sludge (WWS) did not show any toxic effect compared to synthetic sewage. This study provides evidence that CTC and its metal complexes are toxic to bacteria when they are biologically available. As for WWS, CTC was adsorbed to solid part and was not biologically available to show measurable toxic effects.

Key words: Bacteria; Chlortetracycline; Metal complexation; Toxicity; Wastewater sludge.

Introduction

Veterinary usage of antibiotics for growth promotion and feed efficiency enhancement by providing subtherapeutic doses is a flourishing market. Although there is a considerable controversy regarding the necessity of antibiotic use at subtherapeutic doses, still their usage continues. On the other hand, the continued usage of antibiotics has led to emergence of antibiotic resistance (Hayes *et al.*, 2004, Levy, 1978, Libby *et al.*, 1955). Currently, there are no alternatives for antibiotics to control infectious diseases.

Chlortetracycline (CTC) is a broad-spectrum antibiotic against wide range of aerobic and anaerobic Gram-positive and Gram-negative bacteria. CTC is the first antibiotic to show antibacterial (bacteriostatic) activity by inhibiting protein synthesis through the binding of amino acyl-tRNA to the acceptor site on the mRNA-ribosome (Chopra *et al.*, 1992, Schnappinger *et al.*, 1996). Among all tetracycline antibiotics, CTC has lowest absorption (25-30%) with maximum excretion varying from 30-90% among different living organisms (Agwuh *et al.*, 2006, Elmund *et al.*, 1971, Feinman *et al.*, 1978). Hence, maximum unmetabolized CTC ultimately ends up in the environment (wastewater (WW), agriculture field, surface water). CTC is soluble in water but its ability to form complexes with metals results in the formation of less soluble CTC-metal complexes. Further, chelation of CTC with metals changes the chemical properties, such as solubility and partition coefficient. Apart from chelation, its high adsorption capacity leads to retention of CTC in the WWS instead of going into WW. Pulicharla *et al*., 2014 reported higher concentration of CTC in WWS (0.4-0.8 mg/L). Authors correlated higher metals concentration in sludge to CTC accumulation due to its chelation property.

Due to continuous application of biosolids in agriculture or for landfilling, the accumulated CTC in WWS is transported to the soil. In soil, there is a co-existence of CTC and metal ions and their complexes. Hence, the accumulated CTC and its metal complexes in soil may influence the soil microbial community due to their broad-spectrum antibacterial activity. Further, CTC may also leach into groundwater aquifers from soil.

Despite wide application and detection of CTC in different environment (soil, WW, WWS, and surface water), knowledge of metal complexation and adsorption behavior on fate of CTC in WW is limited. Chlortetracycline is known to form complexes with metal ions and shows higher adsorption on sediments, soils and clay materials (Pils *et al.*, 2007, Rivera-Utrilla *et al.*, 2013, Sassman *et al.*, 2005, Wang *et al.*, 2008). Extensive investigation has been carried out on the complexation of tetracyclines with metals (Orth *et al.*, 1999, White *et al.*, 1971). Although numerous studies have focused on the chemistry of the tetracyclines-metal complexation, solution conditions complicate the stoichiometry equilibrium between metals and tetracyclines (Wessels *et al.*, 1998). Complex matrix, such as WW contains macro and micro elements and heavy metals having different ionic potentials, thus exhibiting varied complexation with organic ligands. However, little is known about as to how such a complex matrix affects the CTC-metal equilibrium and further influences their adsorption behavior. Unfortunately, the reported complexation constants of CTC with metals (Ca(II), Mg(II), Cu(II)) and stoichiometry ratios are in strong disagreement. As solution conditions (pH, metal type, and temperature) complicate the stoichiometry equilibrium between metals and tetracyclines (Brion *et al.*, 1986, Couto *et al.*, 2000, Lambs *et al.*, 1988). Hence, it is difficult to use reported stoichiometric CTC-metal equilibrium values for further toxicity evaluation in the bacteria.

Owing to inherent differences in cell wall composition of Gram-positive and Gram-negative bacteria, the toxicity of CTC and its metal complexes may be different. However, this possibility has not been investigated as yet. In this study, *Bacillus thuringiensis* (Bt)*,* an insecticidal Gram positive soil-dwelling bacterium has been selected as model microorganism. Likewise, *Enterobacter aerogenes* (Ea) was selected as model Gram negative bacteria. Toxicity of CTC on microorganisms was evaluated using a rapid, short-term and sensitive biochemical toxicity test at environmentally relevant concentrations.

Overall, the objectives of this study were to: 1) determine the stability constant of CTC-metal complexes of four metal ions Ca(II), Mg(II), Cu(II) and Cr(III); 2) estimation of toxicity of CTC and CTC-metal complexes towards Gram positive and Gram negative bacteria; 3) effect of total solids on the toxicity of CTC towards bacteria grown in synthetic growth media, synthetic sewage (SS), secondary sludge 1 (SS1) and settled secondary sludge 2 (SS2); 4) uptake study of CTC and its metal complexes and; 5) effect of CTC on the metabolic products of bacteria.

Materials and methods

Chemical and reagents

Chlortetracycline was purchased from Toronto Rsearch Chemicals (Toronto, Canada). Calcium chloride anhydrous ($CaCl_2$, >99%), chromium nitrate ($(CrNO_3)_3.9H_2O$, 98%), cupric chloride anhydrous ($CuCl_2$, >99%) and magnesium perchlorate anhydrous $Mg(ClO_4)_2$, >99%) were obtained from Fisher Scientific (New Jersey, USA). Calcium chloride hydrate ($CaCl_2.2H_2O$), potassium dibasic phosphate (K_2HPO_4) and potassium monobasic phosphate (KH_2PO_4, 98%) was supplied by Laboratoire MAT Inc. (Quebec, Canada). Glucose, hydrochloric acid (HCl, 36.5-38%) magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), sodium chloride (NaCl), sodium hydroxide (NaOH, >98%) and urea (>99%) were obtained from Fisher Scientific (Feinman *et al.*). Tryptic soya broth (TSB) was purchased from EMD chemicals Inc. (Darmstadt, Germany). Disodium ethylenediamine tetraacetate (Na_2H_2EDTA, 99%) was purchased from E-bay (Tokyo, Japan). Methanol (HPLC grade, purity >99.8%) and ammonium hydroxide (NH_4OH, 28-30% w/w) were purchased from Fisher Scientific (Ontario, Canada).

Potentiometric Measurements

In this study, four metals were selected; two macro-elements, Ca(II) and Mg(II) having higher importance for the cellular activities and higher abundance in WWS and two heavy metal ions, Cu(II) as trace element for growth of microorganisms and Cr(III) as a toxic metal. The stability constants and stoichiometric equilibrium for binding of the CTC to Ca(II), Mg(II), Cu(II) and Cr(III) metal ions was studied by potentiometric titrations using an automatic potentiometric titrator (T870, Radiometer Analytical, ON, Canada). Experiments were conducted in milliQ water having pH 6.0±0.5 at room temperature similar to WWS pH conditions. The metal ion solutions of Ca(II), Mg(II), Cu(II) and Cr(III) were gravimetrically prepared using anhydrous chloride salts of Ca, Mg and Cu and chromium nitrate in water, respectively. Errors in metal ion concentrations were minimized by using the fresh anhydrous salts and they were completely dissolved in water. Prior to the experiments, CTC was freshly prepared by dissolving it in water. The ionic strength of all working solutions was adjusted to 0.1 mol/L using sodium chloride to minimize potential shifts in pH upon ion binding. All solutions were prepared in Milli-Q water containing residual metal ion concentrations less than 1.0 µg/L. The final pH of all working solutions was adjusted to 6.0 ± 0.5 with NaOH or HCl solutions. The stability constants of CTC were determined by titration of the appropriate solutions of CTC (1×10^{-3} mol/L) in water, using metal solutions as titrant (0.5×10^{-3} mol/L), in 0.1 mol/L NaCl (Şanli *et al.*, 2009). All solutions were purged with nitrogen gas to remove dissolved carbon dioxide in titrant solutions and they were later completely mixed using a magnetic stirrer. The stabilization criterion for the potential readings was 0.2 mV within 60 sec. About 0.5mL/min addition of metal solution was performed by calibrated burette and the titrations were continued, until a constant potential was reached.

WWS sampling and analysis

Secondary sludge samples used in this study were collected from Quebec Urban Community (CUQ) wastewater treatment plant (WWTP) (Sainte-Foy, Quebec City, Quebec, Canada). Samples were stored under dark conditions at 4 ± 1 °C, until used. The sludge was used within a week of its storage to prevent its deterioration. Secondary sludge samples were collected before and after addition of coagulant ($Al_2(SO4)_3$, Al^{3+} content of 48% w/w) for settling of secondary treated sludge. The settling process increased the net concentration of solids (suspended solids) which may affect the contaminants concentration in the sludge as some of them attached (adsorbed) to suspended solids. Different parameters of wastewater sludge samples controlling the growth of bacteria, such as solids content (total solids and total dissolved solids) and pH were determined in accordance using standard methods (AWWA,, 2005).

Synthetic Sewage

Synthetic sewage was prepared according to the OECD guidelines (OECD, 2010) using following composition: peptone (16.0 g/L), beef extract (11.0 g/L), urea (3.0 g/L), NaCl

(0.7 g/L), $CaC1_2.2H_2O$ (0.4 g/L), $MgSO_4.7H_2O$ (0.2 g/L), K_2HPO_4 (2.8 g/L) and pH was adjusted to 6.0±0.5 to meet the real WWS conditions.

Bacterial strains and culture media

Two bacterial strains, Gram-positive, rod-shaped aerobic bacterium *Bacillus thuringiensis* var. kurstaki HD-1 (ATCC 33679) (Bt) and Gram-negative rod-shaped facultative anaerobic bacterium *Enterobacter aerogenes* (NRRL B-407) (Ea) were used as microbial model for CTC toxicity study. Bt was sub-cultured and streaked on tryptic soya agar plates and incubated for 18 h as described by Vidyarthi *et al*., 2002. Ea was also sub-cultured and streaked on tryptic soya agar (TSA) plates (TSA: 3.0% Tryptic Soya Broth (Difco)+1.5% Bacto-Agar (Difco)) and incubated for 48 h at 36±1 $^{\circ}$C and preserved at 4 $^{\circ}$C for future use. The culture media used for Bt growth was a laboratory medium, tryptic soya yeast extract (TSY) [tryptic soya broth (Difco), 3.0% (w/v); yeast extract, 0.3% (w/v)] as mentioned in Vidyarthi et al., (2002). For Ea, modified MS media containing glucose (5.0% (w/v), casein peptone (5.0%), KH_2PO_4 (2.0% (w/v) and yeast extract (0.5%) was used to grow and maintain the microorganism (Sarma et al. 2013). The media pH was adjusted to 6.0 ±0.5 with 0.5 M NaOH or 0.1 N HCl solutions to correlate with the WWS conditions.

Inoculum and culture conditions

A single colony of Bt and Ea from TSA plates was used to inoculate a 50-ml Erlenmeyer flask containing 10 ml of sterilized TSY and modified MS medium, respectively. The flasks were incubated on a rotary shaker at 250 rpm and 30±1 $^{\circ}$C for 12 h and 150 rpm and 37±1 $^{\circ}$C for 12 h for Bt and Ea, respectively. The actively growing cells (in logarithmic growth phase) from these flasks were used as inoculum for the toxicity studies (Vidyarthi *et al.*, 2002). About 2% (v/v) from these flasks was used to inoculate 50-ml Erlenmeyer flasks containing 10 ml of different sterilized media, such as culture media, synthetic sewage, or real secondary sludge samples. For further toxicity studies, these flasks were also incubated under the same culture conditions of respective bacteria, as mentioned earlier. Glassware used for the toxicity studies were previously washed with tap water followed by Milli-Q water and finally dried at 100±0.1 °C for 15 minutes before use.

Toxicity Study

The CTC concentration tested for toxicity was 0.5 mg/L. Pulicharla *et al*., 2014 showed presence of 0.4 to 0.6 mg CTC/kg of sludge sample. Four metals (Ca, Cu, Cr and Mg) were selected to form complexes with CTC to evaluate the toxicity and uptake of CTC and its metal complexes across the cell wall of Bt and Ea bacteria. Chlortetracycline toxicity to Bt and Ea bacteria was measured in terms of colony forming units on agar plates using plate count method (Biesta-Peters *et al.*, 2010). The toxicity studies using these two bacteria were based on the inhibition of protein synthesis by CTC. The end result of toxicity was related to decrease in the metabolic activity of the microorganisms leading to inhibition of microbial growth rate. Bt and Ea were grown in their respective media by using 2 % (v/v) inoculum. Positive and negative controls of the toxicity measurements were performed for CTC and CTC metal complexes. Negative control comprised growth media without CTC and metals. Appropriate positive controls comprised CTC and metals without CTC for CTC-metal complexes toxicity tests. CTC to metal ratio of 1:2, 1:1, 1:1, and 1:1 were used for Ca(II), Mg(II), Cu(II) and Cr(III) metal ions, respectively. The molar ratios of CTC with metals were determined based on the potentiometric titrations.

Investigation of the effect of sludge solids concentration on CTC toxicity

Two different secondary sludge samples (secondary sludge before and after settling) were collected and total and suspended solids concentrations were calculated according to the standard methods (AWWA., 2005). The adsorption capacity of CTC on solids and its characteristic chemical property to form insoluble chelates with metals which explains the higher adsorption of CTC, has been extensively studied in the literature (Rivera-Utrilla *et al.*, 2013, Wang *et al.*, 2008). Therefore, the present investigation was mostly focused on toxicity of CTC adsorbed on sludge solids. For SS, SS1 and SS2 matrix, toxicity studies were performed only with CTC since these matrices contained high metal concentrations and it was difficult to correlate the CTC-metal complexes with toxicity. Rest of the conditions of this toxicity study was similar to those described in the previous section.

Analysis

Viable cell count

At the end of the incubation, 12 h sample for Bt and 12 h sample for Ea was withdrawn and analyzed for colony forming units (CFU) by the serial dilution followed by plating on TSA plates. The appropriately diluted samples of both bacteria were plated on agar plates and incubated for 18 h at 30±1 °C and 48 h at 37±1 °C to form fully developed colonies of Bt and Ea respectively. Two dilutions of each sample were plated. The colonies counted on the plates were between 20 and 300. Each data point in figure is an average of three plate counts for % viability.

CTC uptake studies in Bt and Ea

Uptake studies were performed at 2 h interval for 12 h incubation time for both microorganisms. CTC concentration was measured by using Laser Diode Thermal Desorption (LDTD) coupled with tandem mass spectrometry (Phytronix technologies, Quebec, Canada). A novel method was developed similar to iso-chlortetracycline by LDTD (Pulicharla et al., 2014). The daughter ions identified for CTC in LDTD were 464 and 444 Daltons as reported by many LC-MS/MS methods (Guo *et al.*, 2012, Tong *et al.*, 2009). The samples withdrawn for uptake study at each point during incubation were centrifuged (6708 *x g* for 30 min) and the supernatant was collected to determine the CTC concentration by the developed LDTD method. Clomiphene citrate (1 ng/L) was used as an internal standard.

Gas chromatographic analysis

The analysis of metabolites was performed on a gas chromatograph (GC) (Agilent Technologies, 7890B, USA) equipped with a flame ionization detector. A column temperature gradient of 80-240 ºC was maintained by increasing the temperature at a rate of 20 ºC per minute. GC analysis will help to predict the steps of the bacterial metabolic pathway where CTC can cause inhibition. At the end of 12 h incubation, samples were withdrawn and centrifuged for 10 minutes at 6708 *x g*. The supernatant was collected to determine the metabolite concentration. For all samples, isobutanol was mixed as an internal standard and the injection volume was 1.0 µL (Pachapur *et al.*, 2015).

Statistical Analysis

Comparison of toxicity (% viability) and CTC uptake among CTC and its metal complexes in different media of both bacteria was evaluated statistically using analysis of variance (ANOVA) with SigmaPlote. Data from the three replicates of each experiment were expressed as the mean ± S.D. Difference among CTC-metal complexes toxicity and uptake in Bt and Ea were considered to be statistically significant at $p < 0.05$.

Results and discussion

Complexation of CTC with Ca(II), Mg(II), Cu(II) and Cr(III)

The complexation constants of CTC with Ca(II), Mg(II), Cu(II) and Cr(III) obtained in this study are shown in Table 3.1.1. Complexation studies were performed at pH 6.0±0.5 to understand the binding affinity of CTC in wastewater pH conditions. Although this CTC-metal complexation study may not exactly simulate the complex wastewater conditions, still it is helpful to understand the affinity of CTC complexation with metal ions. Upon comparing the log K values of the complexes, the stability of the complexation species decreased in the order: Mg-CTC > Ca-CTC > Cu-CTC > Cr-CTC. The molar ratio for CTC and Mg(II) and Cu(II) was found to be 1:1, for Cr(III), it was 1:0.6 and for Ca(II), it was 1:2. The difference in the stability constants from previous reports and order of stability is highly dependent on the solution pH and concentration of metal ions as these properties affect the basic ionization constant of CTC and competition among metals for complexation, respectively (Albert, 1953, Lambs *et al.*, 1984). At this pH, 90% of the CTC exists as zwitterion (Gu *et al.*, 2007). These equilibrium ratios were utilized for further toxicity studies of CTC in microorganisms.

Comparison of CTC and its metal complex toxicity in Bt and Ea

In order to test the influence of CTC and its metal complexes on Bt and Ea, acute toxicity studies were conducted. The results of Bt and Ea are depicted in figure 3.1.1. Based on the results, CTC-metal complexes were found to be more toxic than CTC alone. With the current environmental concentration of CTC (0.5 mg/L, Pulicharla *et al.*, 2014), percent viability of the initial bacterial inoculum reduced to 0.22% and 0.08% in

Bt and Ea, respectively. According to the results, the acute toxicity of CTC-metal complexes decreased in following order: Mg-CTC> Cu-CTC>Ca-CTC ≡Cr-CTC for Bt and Mg-CTC ≡ Ca-CTC ≡ Cu-CTC ≡ Cr-CTC for Ea. The standard deviation in plate count for Bt and Ea bacteria was less than 12.0% and 8.4%, respectively.

Chlortetracycline accumulated in both Gram positive and Gram negative bacteria. The differential susceptibility of bacteria is dissimilar for many antibiotics. It is mainly dependent on the cell wall composition and uptake mechanism. Gram positive bacteria lack outer membrane but have thick peptidoglycan layer (murein layer) having high binding capacity and accessibility. However, Gram negative bacteria have thin peptidoglycan layer. Presence of the cell wall polymers, such as lipoteichoic acids and teichoic acids throughout the peptidoglycan layer may be responsible for more susceptibility towards antibiotics in Gram positive bacteria. This can be correlated to the Gram stain retention in the peptidoglycan layer of Gram-positive bacteria. Mutants showed decrease in susceptibility to water-soluble tetracyclines, which provided the evidence that hydrophilic tetracycline drugs enter via the pores in outer membrane of Gram negative bacteria (Leive *et al.*, 1984). Partly diffusion (proton motive force) and energy-dependent carrier-mediated system was responsible for transportation of tetracyclines across the cytoplasmic membrane of bacteria. Bt showed higher susceptibility to CTC-metal complexes than CTC. During control experiments with metals (Ca, Mg, Cu and Cr), Bt showed approximately 20 times higher growth with Mg(II), where this metal ion was essential to fulfill many functions at subcellular sites (Hughes *et al.*, 1973). In the case of Cu(II), growth increased 2-folds, where copper has been regarded as essential trace element to stimulate growth under tested concentrations (Yao *et al*., 2004). With Ca(II), Bt did not show any significant effect ($p > 0.05$) on growth. Cr(III) also did not show any toxic effect on Bt growth, suggesting no uptake of this ion (Beveridge *et al.*, 1976) or no toxic effect at this tested concentration level. In the case of Ea, all these metals did not show any significant effect ($p > 0.05$) on the growth of bacteria at this concentration. Although Mg(II) is essential for many cellular functions, it does not show any effect on the growth of Ea. CTC-metal complexes showed more toxicity in Bt than CTC itself. Among all metal complexes, CTC-Mg showed 40-folds higher toxicity and CTC-Cu complex exhibited 2-folds higher toxicity than CTC alone. This indicated higher uptake of CTC after complexing with Mg(II) and Cu(II) ions. As CTC-Mg complex has higher stability constant at this pH, among all the metals tested, resulting in its higher uptake. This provides evidence that if the metal ion is essential for the bacterial growth, the uptake can happen even if it is in the form of CTC-metal complex. Thus, any antibiotic that was able to complex with essential metal ions may show higher toxicity towards Bt. Experimental results for all metals and CTC together with all four metals did not show any significant effect ($p > 0.05$) on growth promotion and toxicity in Bt, respectively. This may be due to the effect of competition for the binding sites among all metals on murien layer of Bt (Lambert *et al.*, 1975). All CTC-metal complexes in Ea did not show any significant difference ($p > 0.05$) in their toxic effect. Even though CTC and its metal complexes crossed the outer membrane of cell wall (Chopra *et al.*, 1992), yet the complexes may be dissociating in the periplasmic space of cell wall. The free CTC (neutral or zwitterionic) in periplasmic space will cross the cytoplasmic membrane by diffusion through the lipid bilayer regions. Hence, the instability of CTC-metal complexes in the periplasmic space may lead to dissociation of CTC-metal complexes. Thus, there was insignificant difference in their toxicity among CTC and its metal complexes in Ea ($p > 0.05$).

Evaluation of toxicity of CTC in wastewater sludge

Synthetic wastewater sludge

The toxicity of CTC in synthetic wastewater sludge and two sludge samples having different solid concentrations is presented in figure 3.1.2. Compared to conventional synthetic culture media of Bt and Ea, both bacteria showed 5-folds and 40-folds higher growth in synthetic sewage, respectively. This was mainly due to the presence of metal ions in the synthetic sewage. The toxicity of CTC in synthetic sewage was approximately 4.5-folds higher than culture media proportional to growth of Bt in synthetic sewage. Thus, CTC was able to form complexes with metal ions in the synthetic sewage resulting in increased uptake of CTC as CTC metal complex. CTC showed 2-folds higher toxicity in synthetic sewage for Ea. This may be due to higher concentration of CTC-metal complexes crossing the outer membrane along with the free CTC.

Wastewater sludge

Figure 3.1.2 show percent viability of Bt and Ea in real sludge samples, respectively. Growth of Bt and Ea in SS1 and SS2 was lower than the synthetic sewage. It might not directly indicate toxicity of CTC in the sludge. The decreased growth may be due to change in the C and N ratio and lower availability of soluble carbon and nitrogen source from the complex matrix. This will again depend on the metabolic path and ability of bacteria to utilize different carbon sources. The CTC spiked sludge samples with different solid concentrations calculated as shown in the table 3.1.2 were used to estimate the toxicity effect of CTC in the WWS. The collected secondary sludge samples of SS2 contained approximately 10-folds higher suspended solids than SS1. Secondary sludge with lower suspended solids (SS1) showed higher toxicity compared to higher suspended solids (SS2) for Bt and Ea. Higher adsorption capacity of CTC to sludge solids lowered the toxicity. CTC-metal complexation in sludge enhanced the adsorption. Further, CTC can form ternary complexes with organic matter (Lambs *et al.*, 1984). CTC showed faster adsorption and slower desorption rates (Fernández-Calviño *et al.*, 2015), hence the biological accessibility of CTC to the microorganisms was a key factor to determine its antibiotic activity (toxicity).

CTC toxicity in real sludge samples is essential for predicting its biological activity and environmental impact as a toxic contaminant. Moreover, when removal of the trace compounds is performed in WW and WWS, it becomes essential to know if only the compound was removed and that the complex was not left behind, broadening the new understanding of their behavior.

Uptake study of CTC and CTC-metal complexes

CTC uptake by Bt and Ea is presented in figure 3.1.3. Decrease in the concentration of CTC in the supernatant in the first two hours was due to the adsorption of CTC to the bacterial biomass. (Dolliver *et al.*, 2008) reported higher distribution coefficient (K_d) of 1280 L/kg and 2386 L/kg in sandy loamy and clay loamy soils, respectively. Hui *et al.*, (2013) concluded adsorption of CTC on activated sludge as an exothermic process which is thermodynamically favorable. Due to this higher adsorption capacity, many studies have proved that >70% of CTC adsorption takes place within few minutes to two hours (Bao *et al.*, 2009, Dolliver *et al.*, 2008, Guocheng *et al.*, 2012). The significant decrease ($p < 0.05$) in the CTC concentration at 4 h was observed in both Ea and Bt treated with CTC and CTC-metal complexes. Higher uptake in the CTC concentration was detected in CTC-metal complexes compared to CTC alone in Bt. Higher uptake of CTC-Mg metal complex compared to other complexes was detected, whereas CTC uptake concentrations among other CTC-metal complexes showed less significant difference ($p > 0.05$). The higher uptake can also be correlated with the higher toxicity of respective metal complexes. No significant difference ($p > 0.05$) in CTC concentration was observed in Ea in uptake of CTC and its complexes. This CTC uptake in Ea can be compared with the percent viability of Ea where no significant difference ($p>0.05$) was found among CTC and its metal complexes.

Effect of CTC on the metabolic pathway of bacteria

The experimental data of major metabolites of aerobic fermentation such as, acetic acid and ethanol, after 12 and 18 h incubation for Bt and Ea is presented in figure 3.1.4. From figure 3.1.4, it was noted that the reduction in the acetic acid production (>90%) and (>70%) was equivalent to (>90%) and (>65%) higher ethanol production in Bt and Ea, respectively treated with CTC. If glucose metabolism in bacteria was considered, conversion of Acetyl-CoA to acetate pathway yields ATP which serves as a primary energy source of the cell. Likewise, conversion of Acetyl-CoA to ethanol yields NAD^+ which helps in the substrate oxidation and the protection of microorganisms from toxic substances. The inhibition of acetic acid production pathway by CTC resulted in conversion of total Acetyl-CoA to ethanol. The general metabolic pathway and the possible step where CTC inhibited the metabolic path of bacteria are hypothesized in figure 3.1.5. These results supported the fact that CTC was able to inhibit the energy source of bacteria which was needed to synthesize thousands of macromolecules that the cell needs for its growth. ATP also binds to protein and changes it conformations for their expression. mRNA which decodes the protein is responsible for the conversion of Acetyl-CoA to acetate which yields ATP that might be blocked by CTC. In general, bacteria can produce organic acids to protect themselves by chelating the cations present in the surrounding medium (Gadd, 2010). As seen in figure 3.1.3, the concentration of free metal ions was not sufficient to induce the production of acetic acid due to CTC-metal ion complexation. This theory seems to be further helpful for the confirmation of CTC- metal complexation. This study needs further investigation to confirm exact steps where CTC can block the metabolic pathway of microorganisms.

Conclusions

The binding affinity of chlortetracycline with Ca(II), Mg(II), Cu(II) and Cr(III) metal ions showed an order of Mg(II)> Ca(II)> Cu(II)> Cr(III) in the studied pH 6.0±0.5. CTC-metal complexes showed different toxicity in Bt and Ea due to difference in the cell wall structures. CTC-metal complexes demonstrated toxicity in the descending order as follows: Mg-CTC > Cu-CTC > Ca-CTC ≡ Cr-CTC in Bt. The highest toxicity of Mg-CTC complex in Bt was due to higher binding affinity of CTC with Mg(II) and also significant biological role of Mg ions. CTC-metal complexes showed insignificant ($p>0.05$) difference in toxicity for Ea. These toxicity data can be compared with the uptake studies. Lower toxicity of CTC was observed in the real wastewater sludge samples due to the higher adsorption of CTC to the solid part of sludge. Hence, the biological accessibility of CTC to the microorganisms was very low in wastewater sludge containing CTC.

Acknowledgement

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Discovery Grant 355254 and Strategic Grant), and Ministère des Relations internationales du Québec (coopération Québec-Catalanya 2012-2014) for financial support. The views or opinions expressed in this article are those of the authors.

Reference

Agwuh KN & MacGowan A (2006) Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. *Journal of Antimicrobial Chemotherapy* 58(2):256-265.

Albert A (1953) Avidity of terramycin and aureomycin for metallic cations. *Nature* 172:201.

Albert GM, John WF & Michael PS (2002) Chapter 11 Fermentation Pathways In. Microbial Physiology. by Wiley-Liss, ISBN: 0-471-39483-1.cd

Bao Y, Zhou Q & Wang Y (2009) Adsorption characteristics of tetracycline by two soils: assessing role of soil organic matter. *Soil Research* 47(3):286-295.

Beveridge T & Murray R (1976) Uptake and retention of metals by cell walls of Bacillus subtilis. *Journal of Bacteriology* 127(3):1502-1518.

Biesta-Peters EG, Reij MW, Joosten H, Gorris LG & Zwietering MH (2010) Comparison of two optical-density-based methods and a plate count method for estimation of growth parameters of Bacillus cereus. *Applied and environmental microbiology* 76(5):1399-1405.

Brion M, Lambs L & Berthon G (1986) Metal ion-tetracycline interactions in biological fluids. Part 6. Formation of copper (II) complexes with tetracycline and some of its derivatives and appraisal of their biological significance. *Inorganica chimica acta* 123(2):61-68.

Chopra I, Hawkey P & Hinton M (1992) Tetracyclines, molecular and clinical aspects. *Journal of Antimicrobial Chemotherapy* 29(3):245-277.

Couto CM, Montenegro M & Reis S (2000) Tetracycline, oxytetracycline and chlortetracycline complexation with copper (II). Potentiometric study. *Química Nova* 23(4):457-460.

Dolliver H, Gupta S & Noll S (2008) Antibiotic degradation during manure composting. *Journal of environmental quality* 37(3):1245-1253.

Elmund GK, Morrison S, Grant D & Nevins M (1971) Role of excreted chlortetracycline in modifying the decomposition process in feedlot waste. *Bulletin of Environmental Contamination and Toxicology* 6(2):129-132.

Federation WE & Association APH (2005) Standard methods for the examination of water and wastewater. *American Public Health Association (APHA): Washington, DC, USA*.

Feinman SE & Matheson JC (1978) *Draft environmental impact statement: subtherapeutic antibacterial agents in animal feeds.* [Department of Health, Education, and Welfare, Public Health Service], Food and Drug Administration, Bureau of Veterinary Medicine,

Fernández-Calviño D, Bermúdez-Couso A, Arias-Estévez M, Nóvoa-Muñoz JC, Fernández-Sanjurjo MJ, Álvarez-Rodríguez E & Núñez-Delgado A (2015) Kinetics of tetracycline, oxytetracycline, and chlortetracycline adsorption and desorption on two acid soils. *Environmental Science and Pollution Research* 22(1):425-433.

Gadd GM (2010) Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* 156(3):609-643.

Gu C, Karthikeyan K, Sibley SD & Pedersen JA (2007) Complexation of the antibiotic tetracycline with humic acid. *Chemosphere* 66(8):1494-1501.

Guo L, Chen Y, Zhang L, Yang W & He P (2012) Development and validation of a liquid chromatographic/tandem mass spectrometric method for determination of chlortetracycline, oxytetracycline, tetracycline, and doxycycline in animal feeds. *Journal of AOAC International* 95(4):1010-1015.

Guocheng L, Limei W, Xiaolong W, Libing L & Xiaoyu W (2012) Adsorption of chlortetracycline from water by rectories. *Chinese Journal of Chemical Engineering* 20(5):1003-1007.

Hayes JR, English LL, Carr LE, Wagner DD & Joseph SW (2004) Multiple-antibiotic resistance of Enterococcus spp. isolated from commercial poultry production environments. *Applied and Environmental Microbiology* 70(10):6005-6011.

Hughes A, Hancock I & Baddiley J (1973) The function of teichoic acids in cation control in bacterial membranes. *Biochemical Journal* 132(1):83-93.

Lambert PA, Hancock I & Baddiley J (1975) The interaction of magnesium ions with teichoic acid. *Biochemical Journal* 149(3):519-524.

Lambs L, Brion M & Berthon G (1984) Metal ion-tetracycline interactions in biological fluids. Part 3. Formation of mixed-metal ternary complexes of tetracycline, oxytetracycline, doxycycline and minocycline with calcium and magnesium, and their involvement in the bioavailability of these antibiotics in blood plasma. *Inflammation Research* 14(5):743-750.

Lambs L, Decock-Le Reverend B, Kozlowski H & Berthon G (1988) Metal ion-tetracycline interactions in biological fluids. 9. Circular dichroism spectra of calcium and magnesium complexes with tetracycline, oxytetracycline, doxycycline, and chlortetracycline and discussion of their binding modes. *Inorganic Chemistry* 27(17):3001-3012.

Leive L, Telesetsky S, Coleman W & Carr D (1984) Tetracyclines of various hydrophobicities as a probe for permeability of Escherichia coli outer membranes. *Antimicrobial agents and chemotherapy* 25(5):539-544.

Levy SB (1978) Emergence of antibiotic-resistant bacteria in the intestinal flora of farm inhabitants. *The Journal of infectious diseases* 137(5):688-690.

Libby DA & Schaible PJ (1955) Observations on growth responses to antibiotics and arsonic acids in poultry feeds. *Science (Washington)* 121:733-734.

OECD, 2010, guidelines for the testing of chemicals (OECD, 209) 22 July 2010. http://www.biotecnologiebt.it/pdf_our_services/OECD209.pdf.

Orth P, Saenger W & Hinrichs W (1999) Tetracycline-Chelated Mg2+ Ion Initiates Helix Unwinding in Tet Repressor Induction†. *Biochemistry* 38(1):191-198.

Pachapur VL, Sarma SJ, Brar SK, Le Bihan Y, Buelna G & Soccol CR (2015) Evidence of metabolic shift on hydrogen, ethanol and 1, 3-propanediol production from crude glycerol by nitrogen sparging under micro-aerobic conditions using co-culture of Enterobacter aerogenes and Clostridium butyricum. *international journal of hydrogen energy* 40(28):8669-8676.

Pils JR & Laird DA (2007) Sorption of tetracycline and chlortetracycline on K-and Ca-saturated soil clays, humic substances, and clay− humic complexes. *Environmental Science & Technology* 41(6):1928-1933.

Puicharla R, Mohapatra D, Brar S, Drogui P, Auger S & Surampalli R (2014) A persistent antibiotic partitioning and co-relation with metals in wastewater treatment plant—chlortetracycline. *Journal of Environmental Chemical Engineering* 2(3):1596-1603.

Rivera-Utrilla J, Gómez-Pacheco CV, Sánchez-Polo M, López-Peñalver JJ & Ocampo-Pérez R (2013) Tetracycline removal from water by adsorption/bioadsorption on activated carbons and sludge-derived adsorbents. *Journal of environmental management* 131:16-24.

Şanli S, Şanli N & Alsancak G (2009) Determination of protonation constants of some tetracycline antibiotics by potentiometry and lc methods in water and acetonitrile-water binary mixtures. *Journal of the Brazilian Chemical Society* 20(5):939-946.

Sassman SA & Lee LS (2005) Sorption of three tetracyclines by several soils: assessing the role of pH and cation exchange. *Environmental Science & Technology* 39(19):7452-7459.

Schnappinger D & Hillen W (1996) Tetracyclines: antibiotic action, uptake, and resistance mechanisms. *Archives of microbiology* 165(6):359-369.

Tong L, Li P, Wang Y & Zhu K (2009) Analysis of veterinary antibiotic residues in swine wastewater and environmental water samples using optimized SPE-LC/MS/MS. *Chemosphere* 74(8):1090-1097.

Vidyarthi A, Tyagi R, Valero J & Surampalli R (2002) Studies on the production of B. thuringiensis based biopesticides using wastewater sludge as a raw material. *Water research* 36(19):4850-4860.

Wang R, Wei R, Liu T & Wang T (2008) Sorption characteristics of veterinary antibiotics chlortetracycline on manure. *Huan jing ke xue= Huanjing kexue/[bian ji, Zhongguo ke xue yuan huan jing ke xue wei yuan hui" Huan jing ke xue" bian ji wei yuan hui.]* 29(5):1363-1368.

Wessels J, Ford W, Szymczak W & Schneider S (1998) The complexation of tetracycline and anhydrotetracycline with Mg2+ and Ca2+: a spectroscopic study. *The Journal of Physical Chemistry B* 102(46):9323-9331.

White JP & Cantor CR (1971) Role of magnesium in the binding of tetracycline to Escherichia coli ribosomes. *Journal of molecular biology* 58(1):397-400.

Table 3.1.1 Complexation constants of CTC with metals determined in this study

Metal ion	Ca(II)	Mg(II)	Cu(II)	Cr(III)
Log K	3.68±0.36	5.10±0.52	2.77±0.45	0.21±0.78

Table 3.1.2 Different solids content of wastewater sludge used for toxicity study of CTC

Sample ID	pH (±0.5)	Total solids (g/L)	Total dissolved solids (g/L)	Total suspended solids
Synthetic sewage (SS)	6.0	34.1±0.02	34.1±0.03	0.0
Secondary sludge (SS1)	5.8	0.990±0.13	0.500±0.35	0.49±0.24
Settled Secondary sludge (SS2)	6.3	7.127±0.24	1.712±0.21	5.145±0.22

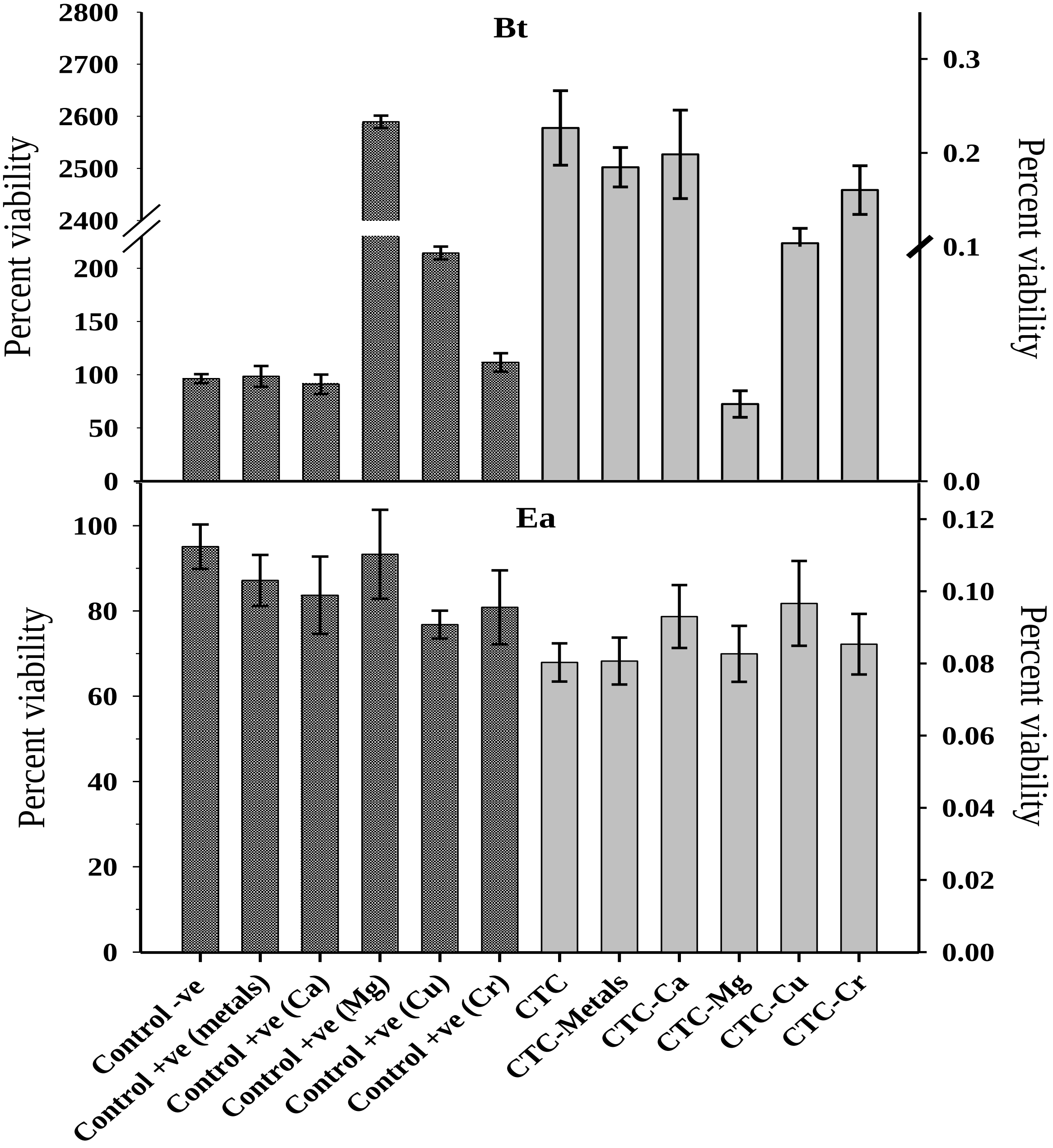


Figure 3.1.1 **Toxicity studies (% viability) of CTC and its metal complexes in *Bacillus thuringiensis* (Bt) and *Enterobacter aerogenes* (Ea). Each data point in figure is an average of three plate counts of percent viability.**

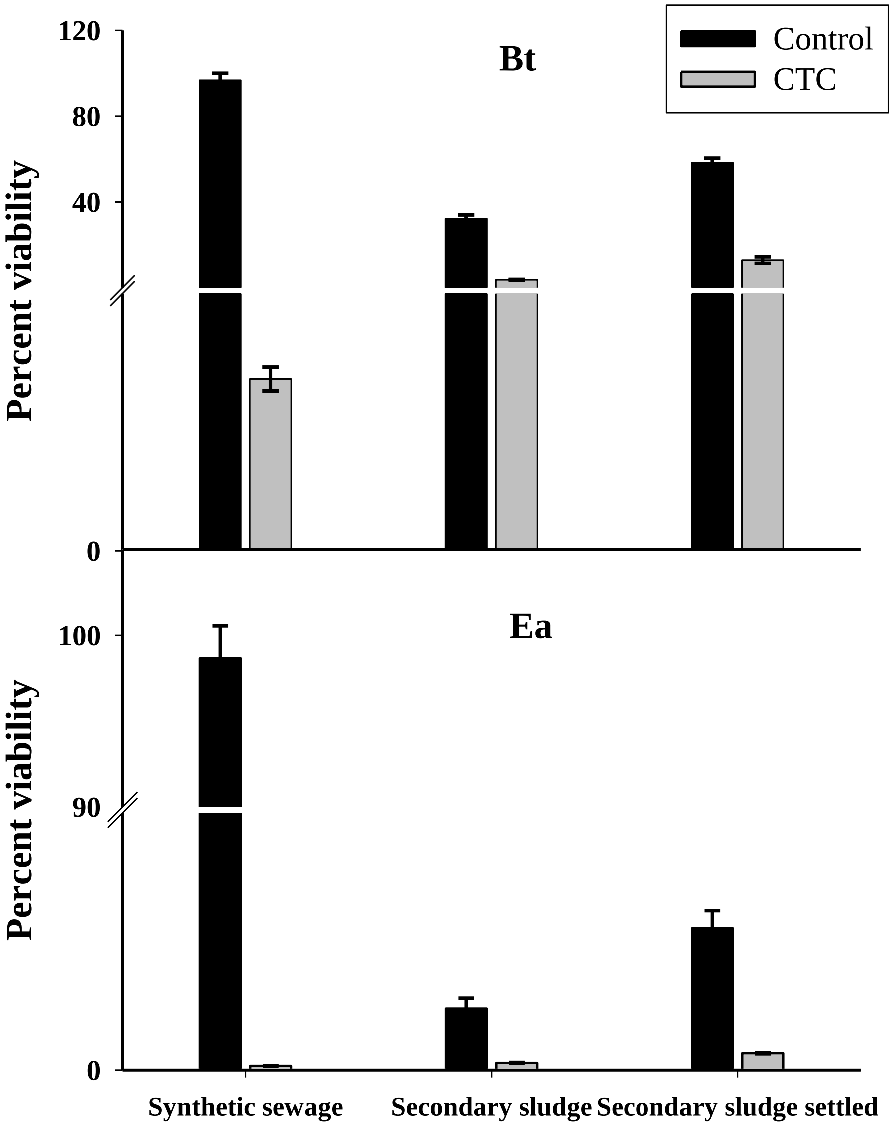


Figure 3.1.2 **Effect of solids concentrations on the toxicity (% viability) of CTC in the synthetic sewage and secondary sludge samples in *Bacillus thuringiensis* (Bt) *and Enterobacter aerogenes* (Ea).**

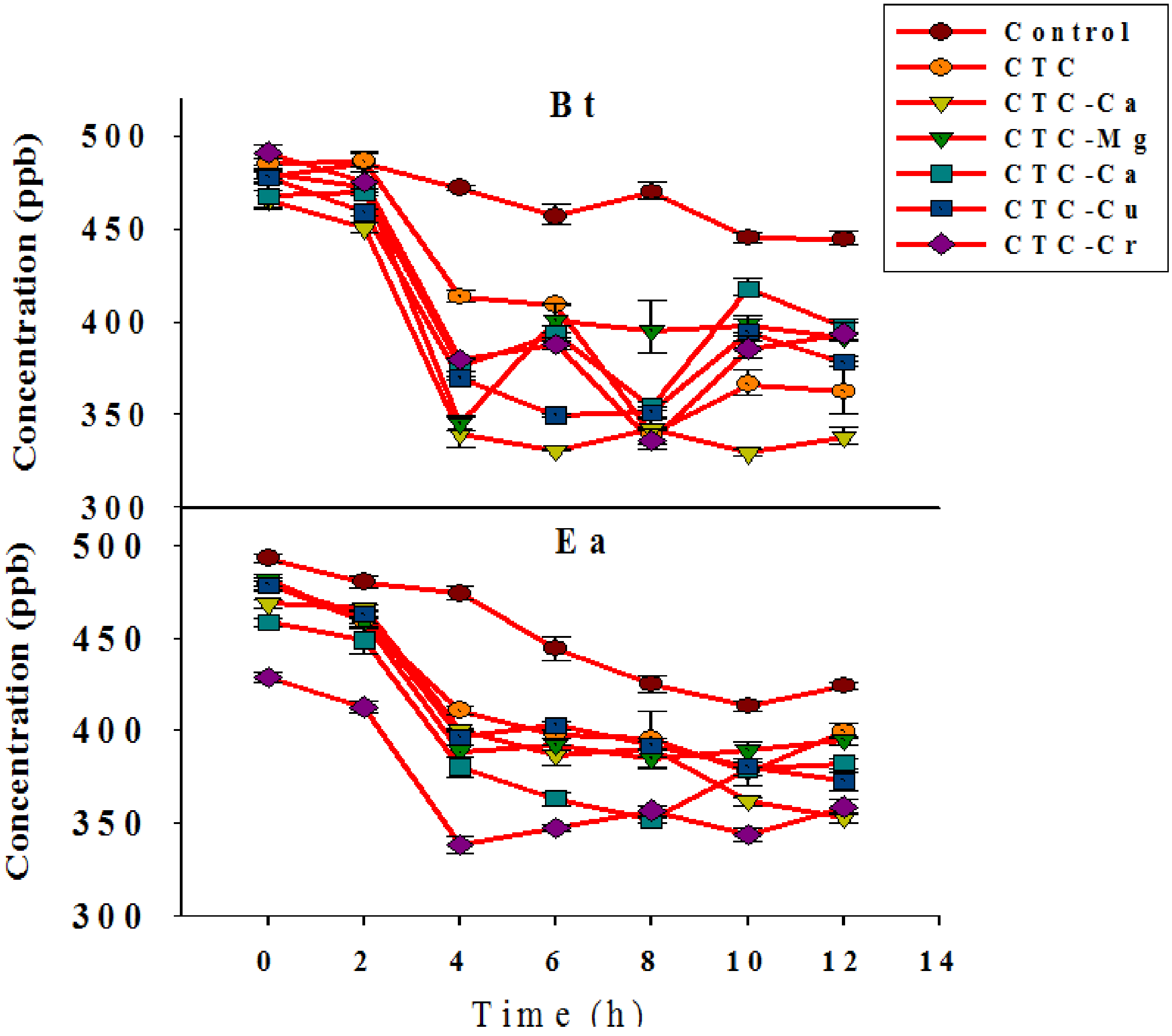


Figure 3.1.3 **Uptake study of CTC and CTC-metal complexes in *Bacillus thuringiensis* (Bt) and *Enterobacter aerogenes* (Ea)**

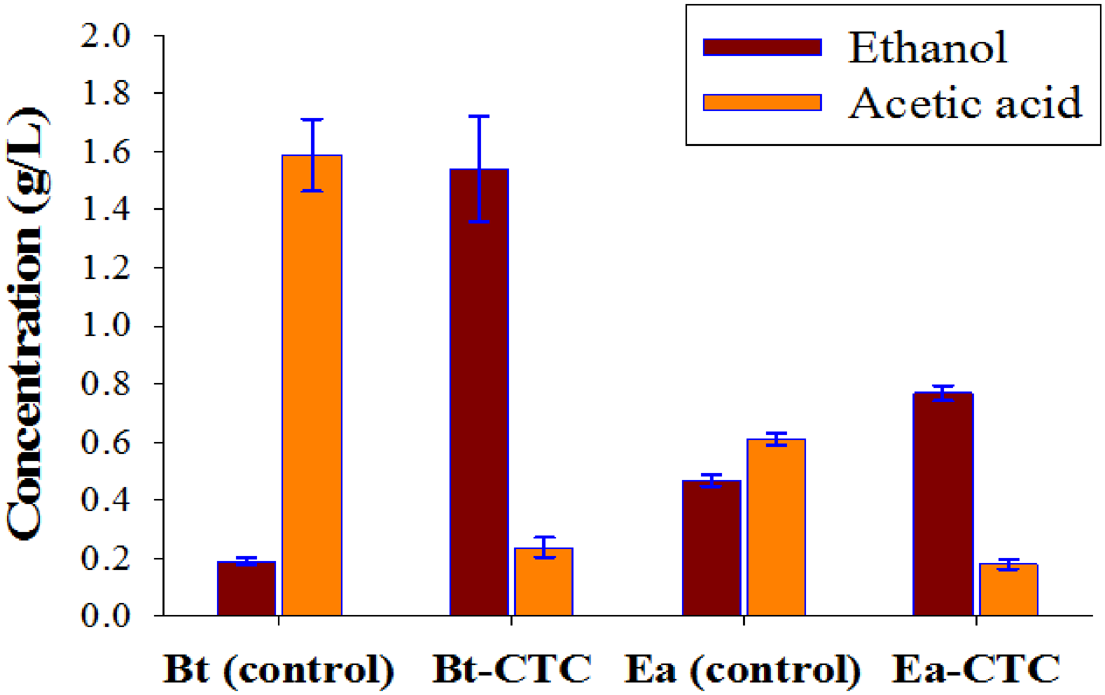


Figure 3.1.4 Effect of CTC on the major metabolites of ***Bacillus thuringiensis*** **(Bt) and** ***Enterobacter aerogenes*** **(Ea) during aerobic fermentation**

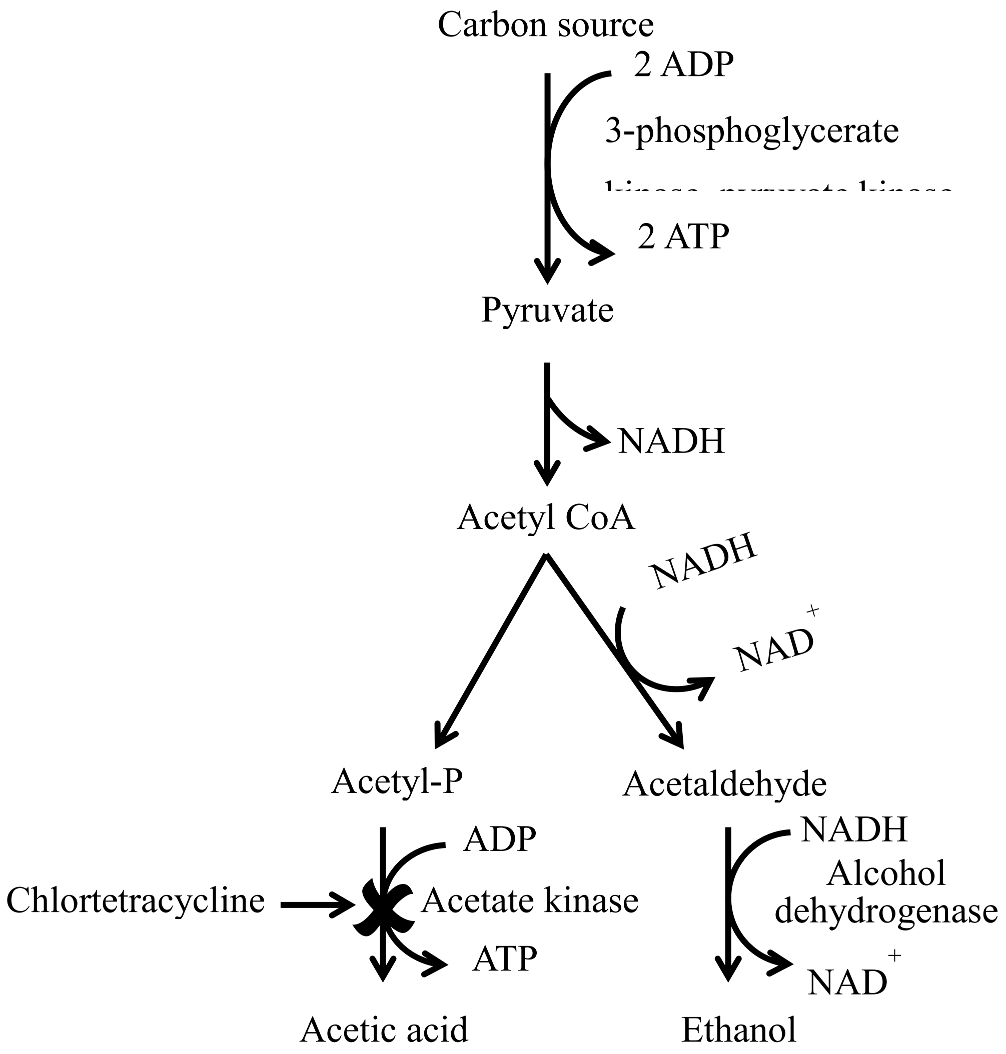


Figure 3.1.5 Proposed blockage step in the general metabolic pathway of bacteria (*Bacillus thuringiensis* and *Enterobacter aerogenes*) where CTC might be exhibiting its bacteriostatic activity.

PART 2

ACUTE IMPACT OF CHLORTETRACYCLINE ON NITRIFYING AND DENITRIFYING PROCESSES

Rama Pulicharla[a], Mehdi Zolfaghari[a], Satinder Kaur Brar[a*], Patrick Drogui[a], Serge Auger[b], Mausam Verma[c], Rao Y. Surampalli[d]

[a] INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9

[b] Phytronix Technologies, 4535 boulevard Wilfrid Hamel, Québec, Canada, G1P 2J7

[c] CO2 Solutions Inc., 2300, Rue Jean-Perrin, Québec, Québec G2C 1T9 Canada

[d] Department of Civil Engineering, University of Nebraska-Lincoln, N104 SEC PO Box 886105, Lincoln, NE 68588-6105, USA

(*Corresponding author, Telephone: + 418 654 3116; Fax: + 418 654 2600; Email: satinder.brar@ete.inrs.ca)

Résumé

Le traitement biologique, en particulier le procédé des boues activées, contribue de manière significative à la récupération des eaux usées. Différents contaminants, en particulier les antibiotiques, affectent négativement les performances des processus de traitement biologique, tels que l'élimination chimique de la demande d'oxygène, la nitrification et la dénitrification. Dans l'étude en cours, la nitrification séquentielle et les expériences anoxiques dans les eaux usées municipales synthétiques ont été exposées à 0,5 à 100 mg/L de chlortétracycline (CTC) pendant 24h pour évaluer l'impact aigu sur les activités microbiennes (suppression d'azote et de carbone organique). Les deux processus ont été significativement ($p<0,05$) inhibés à une concentration de 50 mg/L de CTC et les résultats ont révélé que la nitrification a été affectée par le CTC par rapport au processus anoxique. Dans la nitrification, l'élimination de la DCO et la cinétique d'oxydation de l'ammoniac ont été inhibées à 50% à 10 mg de CTC/L. Et la cinétique d'oxydation des nitrites à 0,5 mg de CTC/L. De même, dans le procédé anoxique, 14 et 10 mg/L de CTC ont inhibé respectivement 50% de l'élimination de la DMS et la cinétique de réduction des nitrates. Dans la nitrification et la dénitrification, 90% de CTC ont été éliminés par adsorption sur des solides de boues. En outre, une concentration plus élevée de CTC dans les effluents anoxiques par rapport aux effluents aérobies indiquait la dissemblance dans la composition des solides des boues, du pH et de la production de biomasse pour les deux processus.

Mots clés: Chlortétracycline; Nitrification; Processus anoxique; Cinétique; Inhibition; Toxicité.

Abstract

Biological treatment, especially activated sludge process plays significant contribution in wastewater reclamation. Different contaminants, especially antibiotics, adversely affect the performance of biological treatment processes, such as chemical oxygen demand removal, nitrification, and denitrification. In the current study, sequential nitrification and anoxic experiments in synthetic municipal wastewater were exposed to 0.5 to 100 mg/L chlortetracycline (CTC) for 24h to evaluate the acute impact on microbial activities (nitrogen and organic carbon removal). Both processes were significantly ($p<0.05$) inhibited at >50 mg/L concentration of CTC and the results revealed that nitrification was adversely affected by CTC compared to anoxic process. In nitrification, COD removal and ammonia oxidation kinetics were inhibited 50% at 10 mg CTC/L. and nitrite oxidation kinetics at 0.5 mg CTC/L. Likewise in anoxic process, 14 and 10 mg/L of CTC inhibited 50% of COD removal and nitrate reduction kinetics, respectively. In nitrification and denitrification, 90% of CTC was removed by adsorbing onto sludge solids. In addition, higher CTC concentration in anoxic effluent compared to aerobic effluents indicated the dissimilarity in the composition of sludge solids, pH and biomass production for both processes.

Keywords: Chlortetracycline; Nitrification; Anoxic process; Kinetics; Inhibition; Toxicity.

Introduction

From the environmental perspective, the high concentration of nitrogen and organic compounds in water sources cause oxygen depletion and eutrophication; which is a major worldwide concern. Above 80% of the municipal wastewater effluent is discharged into the surface water across the world (Ho, 2002). Hence, nutrient balance in wastewater effluent is often given first priority before its release into the surface water (Codd, 2000, Kleinman *et al.*, 2015). Biological wastewater treatment process was reported to be efficient in removing nitrogenous compounds (Wang *et al.*, 2011). In contrast, removal efficiency for trace organic contaminants was reported to be varying with contaminants (Xijuan Chen *et al.*, 2015b, Repice *et al.*, 2013, Salgado *et al.*, 2012). Biological treatment comprises complex microbiological treatment systems which are quite vulnerable for complete nutrients removal from wastewater. Biological nitrogen removal is a two-step process including aerobic autotroph nitrification and anaerobic heterotroph denitrification (Delgado Vela *et al.*, 2015). However, this process is highly susceptible to environmental conditions including pH, temperature, dissolved oxygen and high concentration of toxic chemicals including antibiotics (Katipoglu-Yazan *et al.*, 2015).Further, the microbial strains which are effective in removal of organic contaminants along with biological nitrogen are targeted by bactericidal and bacteriostatic effects depending on the antibiotics which are present in the wastewater. Possible effects of antibiotics on biological nitrogen removal and the fate of microbial cultures in wastewater treatment plants (WWTPs) has been often reported in recent years (Halling-Sørensen, 2001, Matos *et al.*, 2014).

Tetracyclines (TCs) are the main classes of antibiotics that are being widely used in both human and animal infections and also for prophylaxis and growth promoting purposes in animals (Cabello, 2006, Landers *et al.*, 2012, Sarmah *et al.*, 2006). These antibiotics stop the bacterial growth by preventing the binding of aminoacyl-tRNA to the ribosome and thus inhibit the protein synthesis (Chopra *et al.*, 2001). About 30-90% of these TCs were recovered in urine and fecal excretions of animals and humans as active and non-metabolized compounds. Considering these facts, a significant portion of TCs inevitably ended up in the WWTPs. Thus, WWTPs are the main accumulating and point sources for many antibiotics. Chlortetracycline (CTC) is the most commonly used TC for veterinary purposes rather than in humans. Typical CTC concentrations ranging from 0.3-62 µg/L (Karthikeyan *et al.*, 2006, Puicharla *et al.*, 2014) in wastewater and high concentration range 15 µg/kg- 34 mg/kg (Pulicharla *et al.*, 2016, Yuan *et al.*, 2014) in wastewater sludge were detected. Only a few studies reported the impact of CTC on the performance of biological process compared to fate and degradation of CTC in the WWTPs. Further, CTC showed increased and decreased toxicity towards bacteria after metal chelation and adsorption, respectively (Pulicharla *et al.*, 2015). Hence, these properties can affect the biological processes of WWTPs. Studies of Halling et al., 2001 reported the negative effect of CTC on *Nitrosomonas europea* growth in biofilms. However, the impact of CTC on individual dynamics of aerobic and anaerobic processes in WWTP has been not yet reported. Previous studies have only provided the visualization of pulsing inhibitory actions of antibiotics on microbial cultures in a short time exposure (A Chen *et al.*, 2015a, Katipoglu-Yazan *et al.*, 2013).

In particular, autotrophic and heterotrophic fractions of the microbial community might be affected diversely by antibiotics. Hence, the effect of the wide range of CTC concentration on nitrifying and denitrifying bacterial processes was individually studied. The acute impact of CTC was essentially evaluated on the kinetics of biochemical processes including experimental profiles of ammonia-nitrogen (NH_4-N) fractions in nitrification and nitrate-nitrogen (NO_3-N) fractions in anoxic processes. The sensitivity of nitrifying and denitrifying bacteria to CTC was considered as an integral part of evaluation based on the nitrogen and chemical oxygen demand removal efficiency and the growth of biomass. The concentration of CTC was estimated in the effluent filtrate of both processes to understand the removal capacity of CTC by adsorption.

Material and methods

Chemicals

Commercial grade chlortetracycline (99% purity) used in this study was purchased from Toronto Research Chemicals (Toronto, Canada). A stock solution of CTC 1000 mg/L was prepared before starting the experiment by dissolving 1 g in 1 L of Milli-Q water.

$(NH_4)_2SO_4$ (98%), KH_2PO_4 (98%), $MgSO_4$ (99%), $CaCl_2.2H_2O$ (99%), $FeCl_3$ (98%), $CuSO_4.7H_2O$ (98%), $Na_2MoO_4.2H_2O$ (98%), $MnSO_4$ (99%), $ZnCl_2$ (98%), Na_2CO_3 (99.7%), disodium ethylenediaminetetraacetate (Na_2H_2EDTA, 99%), ammonium hydroxide (NH_4OH, 28-30% w/w) and methanol (HPLC grade, purity >99.8%) were purchased from Fisher Scientific (Ontario, Canada). Sodium monobasic phosphate (KH_2PO_4, 99.5%) was supplied by Laboratoire MAT Inc. (Quebec, Canada). Milli-Q/Millli-Ro Milli pore system (Milford, MA, USA) was used to prepare HPLC grade water in the laboratory.

Synthetic wastewater and preparation of synthetic sludge

Synthetic wastewater (SWW) was formulated by taking the average characteristics of Quebec City municipal wastewater, Quebec, Canada as a reference. Composition was as follows with small modifications: glucose 1000±7.43 mg/L, $(NH_4)_2SO_4$ 235.7±3.65 mg/L, KH_2PO_4 61.463±1.5 mg/L, $MgSO_4$ 23.12±0.56 mg/L, $CaCl_2•2H_2O$ 14.67±0.6 mg/L, $FeCl_3$ 7.76±0.82 mg/L, Na_2CO_3 285.74±3.5 mg/L, $CoSO_4· 7H_2O$ 0.32±0.03 mg/L, $Na_2MoO_4•2H_2O$ 0.07±0.08 mg/L, $MnSO_4•H_2O$ 0.08±0.001 mg/L, $ZnCl_2$ 0.15±0.05 mg/L, and $CuSO_4$ 0.05±0.02 mg/L. Aerobic and anoxic activated sludge was developed through membrane sequencing batch reactor (MSBR). Both aerobic and anoxic reactors were inoculated by 1 g/L of membrane batch reactor sludge already developed in previous studies of Zolfaghari et al., 2015. A laboratory scale MSBR consisting of 6 L working volume and an air diffuser for aerobic, and 4 L working volume for anoxic reactor equipped with a magnetic stirrer were operated in a temperature controlled (25±0.5^{o}C) room. Aerobic reactor was operated by filtering 3 L of effluent each day using Zeed-Weed (ZW-1) hollow-fiber membrane manufactured by Zenon Environmental Inc. (Oakville, ON, Canada), followed by feeding with 3L of SWW. For preparing SWW, 320 mL of nitrogen stock solution and 4.8 g of glucose as a source of carbon was added to 2.68 L milliQ water, so as to have 0.8 gCOD/L/day and 0.37 gNH_4-N/L/day. The anoxic reactor was maintained by mixing at 300 rpm for 14 h and after 10 h settling followed by decanting 2 L supernatant and feeding with the same amount of synthetic wastewater. For making anoxic feed, 56 mL of $NaNO_3$ stock solution, and 3.2 g of glucose was dissolved in 2 L of milliQ water which provided 0.8 gCOD and 64.6

mgNOx-N. The dissolved oxygen in aerobic and anoxic reactors was always controlled at 8-9 mg O_2/L and less than 0.2 mg O_2/L, respectively. Sufficient dissolved oxygen was maintained in the aerobic reactor to achieve maximum nitrification in 24h of experiment. Around 500 mL and 125 mL of aerobic and anoxic sludge was removed every three days to maintain sludge retention time (SRT) of 35 days and 100 days, respectively; as well as by monitoring the evolution of total suspended solids (TSS) and total volatile solids (TVS) in both reactors. The following equation was applied in both reactors for measuring the sludge age:

$$SRT = \frac{C_{MLSS}\,V_r}{Q_w\,C_{solid}}$$

where, C_{MLSS} is concentration of mixed liquid suspended solids, Vr is the volume of reactor, C_{solid} is concentration of sludge solids and Q_w is the flow rate of the withdrawal sludge. By considering that the concentration of the total suspended solid in the wastewater was almost constant during experiment, for the aerobic reactor with the volume of 6 L, removal of 500 mL every three days (160 mL/day) was necessary to keep the sludge age at around 35 days. For anoxic reactor with volume of 4 L, removal of 120 mL of sludge per three days (40 mL/day) kept the SRT at 100 days. This high SRT was used to develop steady, stable and sufficient microbial culture for the current experiments. Microbial culture in MSBR was acclimatized for 60 days to reach steady state having 7.9±0.45 g/L TS and 5.3±0.24 g/L VS and 9.9±0.34 g/L TS and 6.5±0.66 g/L VS enabling stable removal of ammonium-nitrogen (NH_4-N) and nitrate-nitrogen (NO_3-N) (over 97%) indicating the stable condition of aerobic and anoxic activated sludge, respectively. It is worth mentioning that glucose is the only source of carbon; hence, in this study, COD and biological oxygen demand (BOD) were almost identical.

Experimental procedure

After stabilization of nitrification and denitrification processes in MSBRs; series of batch experiments were conducted in triplicate to monitor the effects of CTC on the removal of nitrogen and COD. Experiments are performed by withdrawing activated sludge from MSBRs and centrifuging it at 2000 *x g* for 10 min and re-suspending in milliQ water after removal of supernatant. This process was repeated three times to ensure that no residual concentration of nitrogen and COD was left in the supernatant. In this study, six different concentrations of CTC (0.5 mg/L, 5 mg/L 10 mg/L, 25 mg/L, 50 mg/L and 100 mg/L) were studied to evaluate the effect on nitrification and denitrification processes. This wide range of CTC was chosen as low to high concentrations have been detected in different environments, including wastewater sludge (15 µg/kg -34 mg/kg) (Pulicharla and others 2017), animal manure (143.97 mg/kg) (An and others 2015) and soil (108 mg/kg) (Hoese and others 2009). Studies were conducted in 3 L synthetic wastewater having 2 g/L of VSS, and 1000 mgCOD/L; 50 mg/L NH_4-N and 50 mg/L NO_3-N for nitrification and denitrification experiments, respectively. The used 2 g/L of VSS was derived from the centrifuged and re-suspended active sludge and was dispersed in freshly prepared SWW media having mentioned parameters. Each run included 7 batch reactors, one for control to monitor the concentration profiles of nitrogen fractions in the absence of CTC and other six experiments were conducted with the six different CTC concentrations mentioned earlier, to monitor the acute inhibitory effect. CTC impact on microbial activity was monitored by means of NH_4-N and COD removal and generation of NO_3-N in nitrification and NO_3-N and COD removal in denitrification for 24 h. Effect of CTC was observed within 24 h, as control experiments showed maximum (>98%) removal of COD and N within this time period. The sensitivity of both processes was compared based on the kinetics and removal efficiency of NH_4-N and NO_3-N. Inhibition of microbial activity was considered by comparing the removal kinetic profiles of NH_4-N, NO_3-N and COD in control run (absence of CTC) with the tested CTC concentration range. A blank experiment was run as a negative control for nitrification process in water to monitor the ammonia evaporation rate (stripping) in nitrification experiment.

Analytical methods

COD measurements in filtered samples were done by the colorimetric method adopted from standard method ISO 6060 procedure (Zupančič *et al.*, 2012). TSS and VSS were measured by drying the sample at 105 and 550 ˚C, respectively (Rhoades, 1996). The alkalinity of samples was measured by titration with 0.2 N of sulfuric acid adapted from Standard Methods. Ammonium and nitrate were measured using the colorimetric method by LACHAT instrument (QuikChem Method 10-107-06-2-B and 10-115-01-1-B) (ref). CTC in filtered samples was analyzed according to the method developed by

Pulicharla et al., 2014. The analysis was done using LDTD (laser diode thermal desorption) APCI (atmospheric pressure chemical ionization) source (Phytronix Technologies, Quebec, Canada) coupled with triple quad mass spectrometry. All samples and standard solutions were mixed with 0.1 mg/mL EDTA solution for complete thermal desorption of CTC from a bar coded LazWell^{TM} plate which is used to introduce the sample into the LDTD source for analysis. CTC concentration was measured by internal standard (IS) method (clomiphene citrate-5 ng/L). The area ratio of CTC to IS was plotted against the analyte concentration to obtain the calibration curve from 10 to 200 µg/L and determined the CTC concentration in unknown samples. This method has detection limit of 5.13 µg/L and the recovery of 93±2.6% for CTC.

Statistical analysis

All the results were reported as a mean standard deviation and the significance of results was analyzed by analysis of variance (ANOVA) and student's t-test was used to compare between groups with SigmaPlot. P-value < 0.05 was considered to be statistically significant.

Results and discussion

CTC effect on aerobic treatment

COD removal

COD and ammonia removal and nitrate generation with different CTC concentrations of batch experiments are illustrated in figure 3.2.1 (A, B, C). About 99.6% COD and 98.9% NH_4-N removal were achieved for the initial dose of 1000 mg/L COD and 50 mg/L NH_4-N within 24 h in control experiment. However, COD removal efficiency was decreased to 96.8%, 81.3%, 75.5%, 71.2%, 97.6% and 94.2% at 0.5, 5, 10, 25, 50 and 100 CTC mg/L, respectively (figure. 3.2.1A). In control and 0.5 mg CTC/L tests, there was no significant difference ($p > 0.05$) in COD removal at the end of the experiment (24h). Similar results were reported with tetracycline; where 0.2 mg/L concentration did not show any negative effect on nitrification (A Chen *et al.*, 2015a). Apparently, higher concentrations of CTC, such as 50 and 100 mg/L have shown no significant difference ($p > 0.05$) in COD removal. During nitrification, while dissolving these high concentrations of CTC, there was a formation of a huge amount of bubbles and maximum CTC was salted out as insoluble solids from SWW and was also adsorbed to the walls of the reactor. Bubble formation was stopped after 8 h and 12 h during aeration for 50 and 100 mg/L CTC tests, respectively (presented as supplementary data). The presence of inorganic salts, such as ammonium sulfate, sulfates of Mg, Cu, Mn, and Co aid in salting out (fractionation) of CTC from SWW as insoluble solids at pH 7-9 (Charles *et al.*, 1953). Further, low solubility of CTC due to metal chelation and higher adsorption to sludge solids resulted in low bioavailability of CTC to nitrifying bacteria at higher concentrations (50 and 100 mgCTC/L). Hence, there is a deviation in COD removal efficiency at higher CTC concentration compared to lower concentrations. Even though removal efficiency is comparable to control and 0.5 mgCTC/L tests; the rate of COD removal was found to be different for control and different tested CTC concentrations.

Rate constants (K) and half-life ($t_{1/2}$) of COD removal, biological nitrification and alkalinity of conducted nitrification experiments are given in table 3.2.1. CTC effect on nitrification performance followed first order kinetics. In the case of control, 87.9% COD was removed within 2h; hence first 2h data was taken to compare the COD removal rate among the control and tested CTC concentrations. Following COD removal efficiencies were observed with tested CTC concentrations given in parenthesis: 92.1% (0.5), 81.7% (5), 54.8% (10), 35.2% (25), 48.4% (50) and 54.2% (100); and corresponding removal rates are given in table 3.2.1. COD removal rate for 0.5 mgCTC/L was slightly increased to 1.329 mg/L.h compared to control at 1.1125 mg/L.h. Low concentration of CTC (0.5 mg/L) might have produced stress on the microbial cultures of aerobic reactor resulting in increased COD removal rate. Studies reported that tolerant microbes demonstrate higher metabolic activity under various physiological stress conditions compared to sensitive microbes (Cheng *et al.*, 2014). Further increased CTC concentrations showed decreased rate of COD removal. Further, there was no significant difference ($p>0.05$) in the removal rate of COD between 50 and 100 mgCTC/L tests and these two concentrations have shown COD removal rate and efficiency similar to 10 mgCTC/L. These results suggest that at higher concentrations, 10 mg CTC/L might be dissolved in the reactor and the remaining CTC might have salted out from SWW. According to the COD removal, 10 mg/L CTC showed 50% decreased efficiency in nitrification in 2 h. And similar 50% decrease in $t_{1/2}$ of COD removal was also observed for 10 mg CTC /L. There was an initial slight decrease in $t_{1/2}$ in the case of 0.5 mgCTC/L; yet, the further increase in CTC concentration negatively affected COD removal; increased the $t_{1/2}$ to 6.7 times at 25 mgCTC/L. Surprisingly, further increase in CTC enhanced the kinetics of COD removal (Table 3.2.1). The main reason for this adverse phenomenon laid in the maximum CTC salting out as insoluble solids and adsorption to reactor walls as described earlier (see supplementary data, figure 3.2.1). There was no significant difference ($p > 0.05$) in VSS among control, 0.5 and 5 mg/L CTC tests. About 50% reduction of VSS was seen at 10 mg/L CTC similar to COD removal efficiency. A further decreased in VSS was seen with 25 mg/L CTC test.

Effect of CTC on ammonia removal

Limiting factor due to alkalinity on nitrification was excluded by maintaining alkalinity and nitrogen ratio 9.46 in all nitrification experiments (Jin *et al.*, 2015, Sehnem *et al.*, 2013). In contrast to COD removal rate, the effect of CTC on alkalinity was not significant ($p > 0.05$) until 10 mg/L of CTC concentration. Further increase in CTC showed less significant ($p = 0.33$) effect. On the other hand, CTC significantly ($p < 0.05$) decreased the kinetics of biological nitrification similar to COD. NH_4-N concentration was depleted to 98.9% within 9 h in control experiment by ammonia oxidizing bacteria (AOB) (figure 3.2.1B). As the ammonia oxidation was observed to be relatively slower with decreasing the concentration of NH_4-N with time in the reactor; hence first 4 h has been taken into consideration for the rate of NH_4-N depletion to compare with simultaneous generation of nitrate and these two followed the first order kinetics (Table 3.2.1). About 20% of ammonia stripping was observed in first 4 h of negative control nitrification experiment. Control and 0.5 mgCTC/L test did not show any significant ($p > 0.05$) difference in the rate of ammonia depletion. In our previous study (Pulicharla *et al.*, 2015), it was proved that low CTC concentration (0.5 mg/L) in wastewater sludge decreased only 10-25% viability of bacteria. Even though 0.5 mg/L of CTC decreased the 10-25% viability of AOB, due to the high abundance of AOB, the rate of ammonia oxidation was not significantly ($p > 0.05$) affected for 0.5 mgCTC/L compared to control. These results also reflected the fact that AOB might be the dominant bacteria in wastewater compared to nitrite oxidizing bacteria (NOB) (Bellucci *et al.*, 2011, Zeng *et al.*, 2014). However, in the case of nitrite oxidation, this concentration of CTC showed 2 fold reductions in the rate of nitrate generation compared to control. AOB are gram-negative and NOB is the combination of Gram-positive and Gram-negative bacteria (Cébron *et al.*, 2005, Mobarry *et al.*, 1996). It was proved that Gram-positive bacteria were more sensitive to CTC and its metal complexes compared to Gram-negative bacteria (Pulicharla *et al.*, 2015). This gave an indication that nitrite to nitrate oxidation might be the rate limiting step during nitrification in the presence of CTC. This could be due to lower abundance of NOB compared to AOB and also the viability of Gram-positive bacteria in microbial cultures of NOB might have been affected to a larger extent by CTC. Similar results were also observed by Jin et al., 2015 where NOB was more vulnerable to CTC than AOB in livestock wastewater.

Due to strong fluctuations in COD and N removal during nitrification, the time-dependent measurements were taken to get a reliable data on CTC effect and to understand the behavior of COD and N removal during the process. The tested 0.5, 5 and 10 mg/L CTC concentrations, the rate of depletion of NH_4-N was similar to the rate of nitrate generation (figure 3.2.1C). This clearly indicated the effect of CTC on simultaneous depletion of ammonia oxidation and corresponding depletion in the generation of nitrate. For further higher CTC concentration (25 mg/L), 50 folds and 20 folds reduced ammonia oxidation rate and rate of nitrate formation was observed respectively. CTC at 10 and 0.5 mg/L showed 50% reduction in ammonia oxidation and nitrite oxidation rate, respectively. Studies of Mobarry et al., 1996 and Cébron and Garnier, 2005 reported the difference in abundance and composition of AOB and NOB microbial cultures and current study supported their data by showing differential impacts of CTC on these microbial cultures. . Additionally, CTC and its metal complexes have shown different toxicity (susceptibility) towards Gram-positive and Gram-negative bacteria. Hence, it is possible to get different LD_{50} CTC concentrations for AOB and NOB as they have different bacterial composition. It is quite interesting to note that the same antibiotic, CTC has shown different result patterns depending on the type of bacteria and dose of antibiotic. Even high concentrations of contaminants including CTC seem to be naturally rejected through salting out process.

Slight increase in the removal rate of COD and alkalinity at 0.5 mgCTC/L compared to control test indicated that this low CTC concentration caused stress on microbial cultures resulting in increased metabolic activity. This might be having a significant effect on resistance development in microorganisms.

Effect of CTC on anoxic process

Figure 3.2.2 shows the performance of anoxic reactor in the presence of CTC. Only 74.7% of COD removal was observed at the end of the 24h experiment. There was no significant difference ($p > 0.05$) in COD removal for 0.5 (69.1%), 5 (67.6%), and 10 (69.4%) mg/L of tested CTC concentrations in 24 h. Further, tested concentrations have shown 63.0%, 54.3% and 36.7% COD removal for 25, 50 and 100 mg/L respectively. Similar to aerobic process, COD removal in the anoxic process also followed first order kinetics. The rate of COD removal, denitrification and $t_{1/2}$ are given in table 3.2.2. COD removal rate was changed dramatically with an increase in the CTC concentration, especially for concentrations higher than 25 mg/L. The half-life of COD removal was increased from 2 h to almost 16 h in the presence of 100 mg CTC/L. In contrast to COD removal efficiency, control, 0.5 and 5 mg/L tested CTC experiments showed similar ($p > 0.05$) rate of COD removal and 10 mg/L CTC concentration showed 1.4 folds decrease in the rate of COD removal compared to control. The further linear decrease of 3.4 folds was found with 25 mg/L test. Hence the LD_{50} for COD removal might be between 10 and 25 mg/L of CTC and theoretically calculated CTC dose was around 14 mg/L. However, 50 and 100 mg/L dose of CTC showed different COD removal rates compared to other concentrations. CTC at 50 mg/L showed 1.4 folds increase and 100 mg/L showed 4 folds decrease in denitrification compared to 25 mg/L. At higher concentration of CTC under anoxic conditions, slow salting out of CTC was observed for 6h in both concentrations. Slow salting out at 50 mg/L of CTC decreases its bioavailability and increases the COD removal efficiency compared to 25 mg/L CTC. But for 100 mg/L, similar slow salting out, but more bioavailability of CTC might be responsible for 4 folds decreased COD removal rate compared to 25 mg/L CTC.

Continuous reduction of VS was seen in anoxic sludge similar to decrease in NO_3-N removal efficiency.

NO_3-N removal efficiency from 89.1% (control) was reduced to 86.6%, 81.6%, 75.8%, 68.2%, 56.6% and 14.6% for 0.5, 5, 10, 25, 50 and 100 mg/L CTC tests, respectively in 3h of experiment. About 0.5 mg CTC/L did not significantly ($p > 0.05$) affect the denitrification efficiency. Similar to COD removal, denitrification half-life was gradually increased from 0.9 h to 9.7 h until 100 mg CTC/L. For nitrate removal rate, 10 mg CTC /L showed LD_{50} for the rate of NO_3-N removal. By comparing both COD and NO_3-N rates of removal, it was observed that NO_3-N removal rate was approximately double the COD removal rate. Studies have proved that nitrate removal rate depends on the availability of carbon source (Osaka and others 2008). In this study, glucose is the only carbon source. Hence, carbon source is not the limiting factor in anoxic process and further it was found that CTC showed more significant ($p < 0.05$) effect on nitrate removal than COD. and . The small difference in LD_{50} for COD and NO_3-N removal might be due to simultaneous utilization of nitrate and organic carbon sources in anoxic process (Modin *et al.*, 2007). Even though COD and nitrate removal was simultaneous, in the presence of CTC, the bacteria might have utilized the available carbon source under stress.

Denitrification needs further studies to confirm the fact that changes in the COD source can affect the nitrate removal efficiency in the presence of CTC.

Comparison of nitrification and denitrification

Table 3.2.3 shows COD and NH_4-N removal efficiency during nitrification and NO_3-N removal efficiency of denitrification. Nitrification seemed to be more susceptible to CTC compared to denitrification if COD removal efficiency was considered. The higher inhibitory effects on nitrification compared to anoxic process were also reported in the previous studies of (Pell *et al.*, 1998) in the presence of other organic and inorganic contaminants. And also inhibition of bacterial growth by preventing the association of aminoacyl-tRNA with ribosomal acceptor (A) site of bacteria by CTC also denotes that aerobic system is affected more than an anoxic system. In the case of anoxic system, first three CTC concentrations did not show any significant ($p > 0.05$) difference in COD removal and 10% decrease in the COD removal were seen for these concentrations compared to control. And for higher CTC concentration tests, COD removal efficiency continuously decreased to 49% until 100 mg/L. On the other hand, during nitrification, 0.5 mg/L CTC did not show any significance ($p > 0.05$) in COD removal and further increased CTC concentrations showed continuous decreased to 72% efficiency until 25 mg/L CTC dose. In contrast, 50 and 100 mg/L CTC tests showed similar COD removal efficiency as the control. This might be due to higher salting out of CTC in the aerobic reactor which leads to its lower bioavailability to affect the COD removal. Even though higher concentrations of dissolved CTC was detected in the effluent of 50 and 100 mg/L CTC tests compared to lower CTC concentrations (Table 3.2.3), lower CTC concentrations showed inhibitory effects on biological treatment process when compared to higher concentrations which took longer time for solubilization (24 h) and hence causing effects. On comparing the process efficiency in 2 h, > 50 mg/L CTC concentrations showed similar effect as 10-25 mg/L CTC concentrations. On the other hand, the aeration enhanced the salting out of CTC in aerobic reactor whereas lower salting out was observed in anoxic reactor. Hence, salting out might be responsible for disagreement of results between nitrification and denitrification at higher CTC concentrations. Since biomass production in the aerobic system is higher compared to the anoxic system and it was established that CTC inhibits the growth of bacteria by preventing the association of aminoacyl-tRNA with ribosomal acceptor (A) site of bacteria; this inhibits protein synthesis. Thus, aerobic system is affected more than an anoxic system. The rate of NO_3-N formation seemed to be higher compared to NH_4-N depletion in control and also nitrite oxidation is more sensitive to CTC compared to NH_4-N depletion indicating that nitrite to nitrate oxidation might be the rate limiting step in nitrification in the presence of CTC.

Detected CTC concentrations of all experiments are given in table 3.2.3 for both nitrification and denitrification experiments. Above 90% of CTC was adsorbed in all experiments. Similar results were reported in the literature that high adsorption and decreased solubility due to metal complexation makes it possible to remove CTC above 90% in WWTPs. With the increase in the concentration of CTC, increased CTC was detected in the effluent. And the higher concentration of CTC has found in anoxic effluents compared to aerobic. This might be due to the difference in the aerobic and anoxic sludge composition (Eckenfelder *et al.*, 1988) and pH. pH of nitrification effluent decreased from 8.3 to 7.2 due to formation of acid by-products during nitrification for all experiments (Anthonisen *et al.*, 1976). However in denitrification, pH decreased from 7.5 to 6.5eventhough the process produced alkalinity, this might be due to accumulation of nitrous oxide (N_2O) in the anoxic process or formation of volatile organic acids (Oguz, 2004). . CTC exists as zwitterion between pH 5-7 and in anoxic effluent below pH 7, the zwitterionic CTC has a low tendency to form metal complexes and lower adsorption to sludge. And also the low biomass production in anaerobic reactor led to higher concentrations of CTC detection in the anoxic effluent of CTC test compared to aerobic effluent. Considering the reported biodegradation of CTC in many studies including manure composting and wastewater (Arikan 2008); no biodegradation of CTC was considered in the studied experimental timeframe.

The results indicated that 50% of nitrogen removal stopped at 10 mg/L of CTC in wastewater. Thus, the current increasing concentration of CTC in wastewater definitely affects the nitrogen removal efficiency in WWTP.

Conclusions

Effect of CTC on nitrification and anoxic process was individually evaluated. Results were highly significant ($p < 0.05$) in differentiating the inhibitory effect of CTC on aerobic and anoxic processes of wastewater. Different inhibitory impacts on nitrification and anoxic systems were principally kinetic driven. CTC at 10 mg/L inhibited 50% of COD removal and ammonia oxidation. And at 0.5 mg/L of CTC, nitrite oxidation was 50 % inhibited. Hence, nitrite to nitrate oxidation was more susceptible than ammonia oxidation during nitrification. And 14 and 10 mg/L of CTC inhibited COD removal and nitrate reduction kinetics, respectively during anoxic process. Different microbiological cultures and their abundance were responsible for the caried LD_{50} for both processes and the results concluded that nitrification bacteria were more sensitive to CTC compared to denitrification. High concentrations of CTC was detected in anoxic effluent compared to aerobic effluent due to the difference in the composition of sludge solids and pH. Low to high (0.5-25 mg/L) concentration of CTC inhibited varied processes of nitrification and anoxic process during biological nitrogen removal.

Aknowledgement

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Discovery Grant 355254 and Strategic Grant), and Ministère des Relations internationals du Québec (coopération Québec-Catalanya 2012-2014) for financial support. Authors are also thankful to “merit scholarship program for foreign students” (FQRNT) for financial assistance to Rama Pulicharla. The views or opinions expressed in this article are those of the authors. Authors are sincerely thankful to Ouarda Yassine for helping in the production of sludge.

References

Anthonisen A, Loehr R, Prakasam T & Srinath E (1976) Inhibition of nitrification by ammonia and nitrous acid. *Journal (Water Pollution Control Federation)*:835-852.

Bellucci M, Ofiţeru ID, Graham DW, Head IM & Curtis TP (2011) Low-dissolved-oxygen nitrifying systems exploit ammonia-oxidizing bacteria with unusually high yields. *Applied and environmental microbiology* 77(21):7787-7796.

Cabello FC (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental microbiology* 8(7):1137-1144.

Cébron A & Garnier J (2005) Nitrobacter and Nitrospira genera as representatives of nitrite-oxidizing bacteria: Detection, quantification and growth along the lower Seine River (France). *Water Research* 39(20):4979-4992.

Charles P & Starbird EE (1953) Recovery of chlortetracycline. (Google Patents).

Chen A, Chen Y, Ding C, Liang H & Yang B (2015a) Effects of tetracycline on simultaneous biological wastewater nitrogen and phosphorus removal. *RSC Advances* 5(73):59326-59334.

Chen X, Vollertsen J, Nielsen JL, Dall AG & Bester K (2015b) Degradation of PPCPs in activated sludge from different WWTPs in Denmark. *Ecotoxicology* 24(10):2073-2080.

Cheng D & He Q (2014) Assessment of environmental stresses for enhanced microalgal biofuel production—an overview. *Frontiers in Energy Research* 2:26.

Chopra I & Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and molecular biology reviews* 65(2):232-260.

Codd GA (2000) Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering* 16(1):51-60.

Delgado Vela J, Stadler LB, Martin KJ, Raskin L, Bott CB & Love NG (2015) Prospects for Biological Nitrogen Removal from Anaerobic Effluents during Mainstream Wastewater Treatment. *Environmental Science & Technology Letters* 2(9):234-244.

Eckenfelder WW, Patoczka J & Pulliam G (1988) Anaerobic versus aerobic treatment in the USA. *Proceedings of the 5th international symposium on anaerobic digestion.*

Halling-Sørensen B (2001) Inhibition of aerobic growth and nitrification of bacteria in sewage sludge by antibacterial agents. *Archives of Environmental Contamination and Toxicology* 40(4):451-460.

Ho G (2002) International Sourcebook on Environmentally Sound Technologies for Wastewater and Stormwater Management. *Technical Publication* 15.

Jin S, Liu M, Chen Y & Liu Y (2015) Combined Effects of Alkalinity Limitation and Chlortetracycline Addition on Ammonia Removal in Sequencing Batch Reactors. *Environmental Engineering Science* 32(10):899-906.

Karthikeyan K & Meyer MT (2006) Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Science of the Total Environment* 361(1):196-207.

Katipoglu-Yazan T, Merlin C, Pons M-N, Ubay-Cokgor E & Orhon D (2015) Chronic impact of tetracycline on nitrification kinetics and the activity of enriched nitrifying microbial culture. *Water Research* 72:227-238.

Katipoglu-Yazan T, Pala-Ozkok I, Ubay-Cokgor E & Orhon D (2013) Acute impact of erythromycin and tetracycline on the kinetics of nitrification and organic carbon removal in mixed microbial culture. *Bioresource technology* 144:410-419.

Kleinman PJ, Sharpley AN, Withers PJ, Bergström L, Johnson LT & Doody DG (2015) Implementing agricultural phosphorus science and management to combat eutrophication. *Ambio* 44(2):297-310.

Landers TF, Cohen B, Wittum TE & Larson EL (2012) A review of antibiotic use in food animals: perspective, policy, and potential. *Public health reports*:4-22.

Matos M, Pereira MA, Parpot P, Brito AG & Nogueira R (2014) Influence of tetracycline on the microbial community composition and activity of nitrifying biofilms. *Chemosphere* 117:295-302.

Mobarry BK, Wagner M, Urbain V, Rittmann BE & Stahl DA (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Applied and Environmental Microbiology* 62(6):2156-2162.

Modin O, Fukushi K & Yamamoto K (2007) Denitrification with methane as external carbon source. *Water Research* 41(12):2726-2738.

Oguz M (2004) Volatile Fatty Acids Effects on Nitrite Removal and Nitrate Formation during Activated Sludge Treatment.

Pell M, Stenberg B & Torstensson L (1998) Potential denitrification and nitrification tests for evaluation of pesticide effects in soil. *Ambio*:24-28.

Puicharla R, Mohapatra D, Brar S, Drogui P, Auger S & Surampalli R (2014) A persistent antibiotic partitioning and co-relation with metals in wastewater treatment plant—Chlortetracycline. *Journal of Environmental Chemical Engineering* 2(3):1596-1603.

Pulicharla R, Brar SK, Rouissi T, Auger S, Drogui P, Verma M & Surampalli RY (2016) Degradation of chlortetracycline in wastewater sludge by ultrasonication, Fenton oxidation, and ferro-sonication. *Ultrasonics Sonochemistry*.

Pulicharla R, Das RK, Brar SK, Drogui P, Sarma SJ, Verma M, Surampalli RY & Valero JR (2015) Toxicity of chlortetracycline and its metal complexes to model microorganisms in wastewater sludge. *Science of The Total Environment* 532:669-675.

Repice C, Dal Grande M, Maggi R & Pedrazzani R (2013) Licit and illicit drugs in a wastewater treatment plant in Verona, Italy. *Science of the Total Environment* 463:27-34.

Rhoades J (1996) Salinity: Electrical conductivity and total dissolved solids. *Methods of Soil Analysis Part 3—Chemical Methods* (methodsofsoilan3):417-435.

Salgado R, Marques R, Noronha J, Carvalho G, Oehmen A & Reis M (2012) Assessing the removal of pharmaceuticals and personal care products in a full-scale activated sludge plant. *Environmental Science and Pollution Research* 19(5):1818-1827.

Sarmah AK, Meyer MT & Boxall ABA (2006) A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* 65(5):725-759.

Sehnem NT, da Silva Machado A, Leite FCB, de Barros Pita W, de Morais MA & Ayub MAZ (2013) 5-Hydroxymethylfurfural induces ADH7 and ARI1 expression in tolerant industrial Saccharomyces cerevisiae strain P6H9 during bioethanol production. *Bioresource technology* 133:190-196.

Wang B & Lan CQ (2011) Biomass production and nitrogen and phosphorus removal by the green alga Neochloris oleoabundans in simulated wastewater and secondary municipal wastewater effluent. *Bioresource technology* 102(10):5639-5644.

Yuan X, Qiang Z, Ben W, Zhu B & Liu J (2014) Rapid detection of multiple class pharmaceuticals in both municipal wastewater and sludge with ultra high performance liquid chromatography tandem mass spectrometry. *Journal of Environmental Sciences* 26(9):1949-1959.

Zeng W, Bai X, Zhang L, Wang A & Peng Y (2014) Population dynamics of nitrifying bacteria for nitritation achieved in Johannesburg (JHB) process treating municipal wastewater. *Bioresource technology* 162:30-37.

Zupančič GD & Roš M (2012) Determination of chemical oxygen demand in substrates from anaerobic treatment of solid organic waste. *Waste and Biomass Valorization* 3(1):89-98.

Table 3.2.1 Effect of different concentrations of CTC on the constant rate of pseudo-first order kinetics and half-life of COD removal, biological nitrification, and alkalinity removal in nitrification (Initial concentration: 1000 mg COD/L, 50 mg NH_4/L, 2100 mgTS/L, 1700 mgVS/L, pH=7.2±0.2, temperature of 20 ˚C).

			CTC concentration (mg/L)					
	Parameter	**0**	**0.5**	**5**	**10**	**25**	**50**	**100**
COD Removal (2 h)	**K (1/h)**	1.113±0.13	1.33±0.07	0.85±0.04	0.39±0.03	0.17±0.012	0.35±0.04	0.41±0.07
	R^2 (%)	98.6±2.6	98.8±1.98	96.2±1.6	97.7±3	77.6±1.8	98.2±5.1	95.5±2.7
	$t_{1/2}$ (h)	0.62±0.06	0.52±0.06	0.82±0.06	1.77±0.16	4.2±0.2	1.99±0.29	1.7±0.42
Biological Nitrification (4 h)	**K (1/h)**	0.588±0.08	0.349±0.04	0.26±0.03	0.22±0.04	0.15±0.1	0.14±0.03	0.13±0.08
	R^2 (%)	97.4±2.4	86.7±0.92	96.8±3.4	80±3.9	97.4±2.5	96.9±3.2	93.6±0.9
	$t_{1/2}$ (h)	1.18±0.08	1.98±0.16	2.7±0.2	3.19±0.22	4.65±0.2	4.9±0.33	5.17±0.53
Alkalinity Removal (9 h)	**K (1/h)**	0.2±0.07	0.24±0.04	0.19±0.04	0.180.06	0.16±0.04	0.13±0.77	0.16±0.04
	R^2 (%)	96.04±0.7	91.6±3.2	89.3±2	92.2±6	78.14±2.9	96.9±6.2	81.6±4.8
	$t_{1/2}$ (h)	4.91±0.06	4.2±0.55	5.14±0.54	5.69±0.9	6.4±0.5	7.6±0.5	6.4±0.69

K: Rate constant; R^2: coefficient; $t_{1/2}$:half life

Table 3.2.2 Effect of different concentrations of CTC on the constant rate of pseudo-first order kinetics and half-life of COD and NO_3-N removal in denitrification (Initial concentration: 1000 mg COD/L, 50 mg NO_3/L, 2100 mgTS/L, 1700 mgVS/L, pH=7.2±0.2, temperature of 20 ˚C).

		CTC concentration (mg/L)						
	Parameter	**0**	**0.5**	**5**	**10**	**25**	**50**	**100**
COD Removal (2 h)	K (1/h)	0.347±0.02	0.33±0.01	0.289±0.01	0.25±0.02	0.123±0.03	0.13±0.02	0.044±0.01
	R^2 (%)	95.7±1.15	91.7±0.92	85.5±1.06	92.1±1.48	97.4±1.7	94.9±2.1	94.2±1.98
	$t_{1/2}$ (h)	2.0±0.01	2.09±0.06	2.40±0.14	2.77±0.11	5.63±0.12	5.21±0.03	15.9±0.8
Biological Denitrification (3 h)	K (1/h)	0.77±0.02	0.69±0.03	0.606±0.07	0.34±0.04	0.32±0.05	0.25±0.03	0.07±0.01
	R^2 (%)	94.4±1.13	94.4±1.13	95.9±0.78	99.1±2.19	90.8±2.7	95.6±2.6	98.3±2.33
	$t_{1/2}$ (h)	0.9±0.02	1.0±0.03	1.14±0.11	2.04±0.25	2.15±0.11	2.76±0.1	9.7±0.2

K: Rate constant; R^2: coefficient; $t_{1/2}$:half life

Table 3.2.3 Comparison data of nitrification and denitrification on exposure to chlortetracycline (CTC)

CTC tested concentration (mg/L)	COD nitrification (%) (24h)	COD denitrification (%) (24h)	NH_4^+-N removal (%) (3h)	NO_3^--N removal (%) (3h)	CTC in effluent of nitrification (µg/L)	CTC in effluent of denitrification (µg/L)
control	97±3.5	101±2.4	95±7.2	99±2.6	0.48±1.1	0.5±4.1
0.5	97.2±5.9	92.5±3.5	96.1±1.7	97.2±2.8	12.95±5.4	9.4±8.6
5	81.6±1.9	90.5±3.3	89.0±1.8	91.7±6.1	44.7±5.1	91.4±2.1
10	75.8±2.7	92.9±3.9	76.0±6.3	85.1±4.2	604.2±3.2	588.4±5.3
25	71.5±5.8	84.3±5.1	67.2±5.2	76.6±5.7	652.6±5.7	1618.1±4.4
50	98.0±4.1	72.6±2.2	53.5±3.5	63.6±5.5	1533.5±2.8	2656.0±5.1
100	94.7±1.1	49.1±4.8	79.9±4.2	16.4±2.7	2379.5±4.5	4175.0±5.4

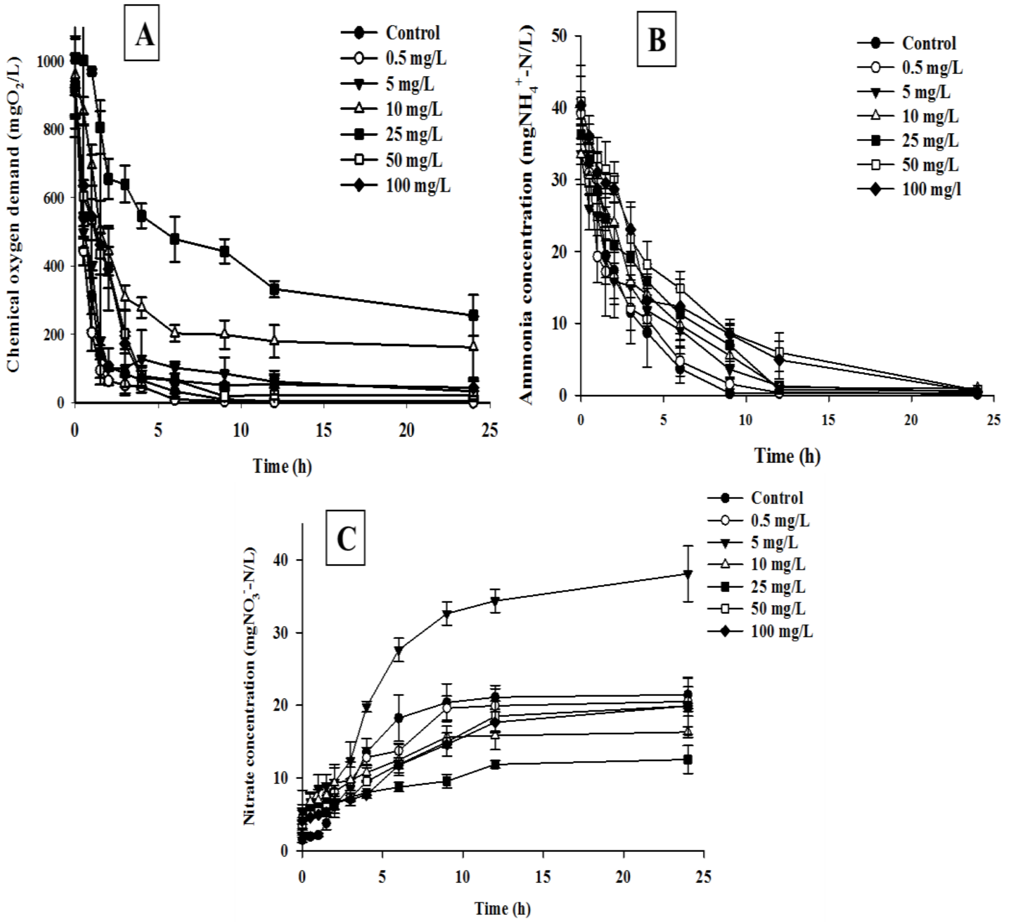


Figure 3.2.1 **Nitrification performance in the presence of CTC tested range in aerobic reactor; A) COD removal; B) ammonia removal; and C) nitrate generation.**

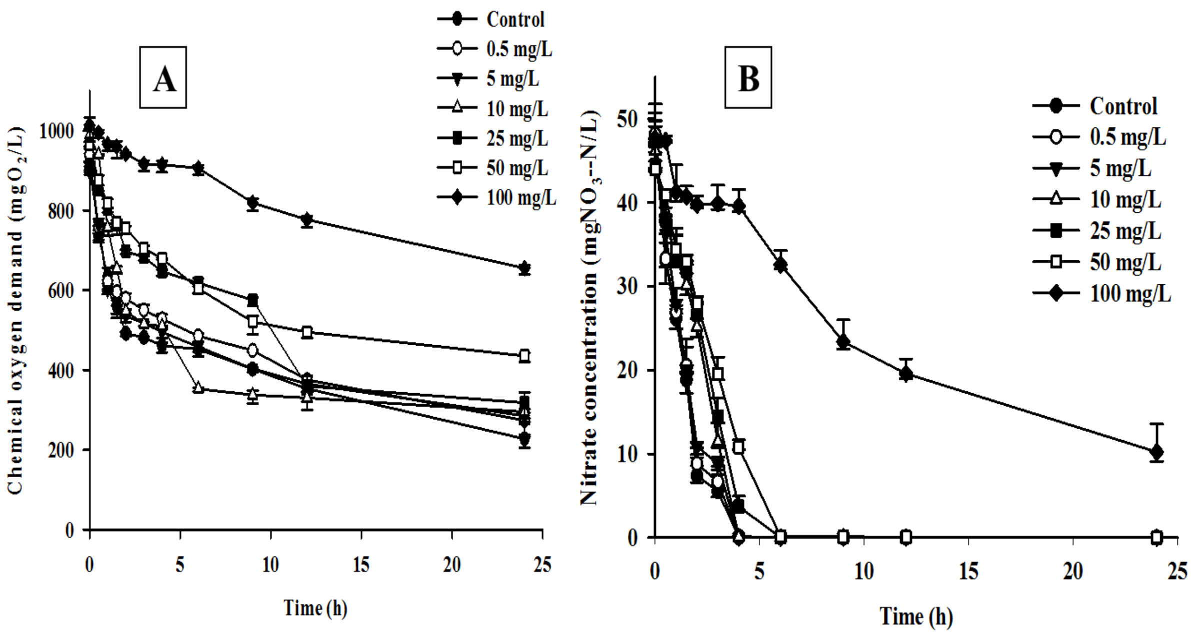


Figure 3.2.2 **Denitrification performance in the presence of CTC on aerobic reactor; A) COD removal and B) nitrate removal.**

CHAPTER 4

HYBRID AOPS FOR DEGRADATION OF CTC

PART 1

DEGRADATION OF CHLORTETRACYCLINE IN WASTEWATER SLUDGE BY ULTRASONICATION, FENTON OXIDATION, AND FERRO-SONICATION

Rama Pulicharla[a], Satinder Kaur Brar[a*], Tarek Rouissi[a], Serge Auger[b], Patrick Drogui[a], Mausam Verma[c], Rao Y. Surampalli[d]

[a] INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9.

[b] Phytronix Technologies, 4535 boulevard Wilfrid Hamel, Québec, Canada, G1P 2J7

[c] CO2 Solutions Inc., 2300, Rue Jean-Perrin, Québec, Québec G2C 1T9 Canada.

[d] Department of Civil Engineering, University of Nebraska-Lincoln, N104 SEC PO Box 886105, Lincoln, NE 68588-6105, USA.

* Corresponding author: INRS-ETE, E-mail: satinder.brar@ete.inrs.ca; Téléphone: + 418 654 3116; Fax: + 418 654 2600.

Ultrasonics Sonochemistry (2017) 34 :332-342

Résumé

Les contaminants résiduels émergents dans les boues d'eaux usées restent un obstacle à ses applications larges et sûres telles que la mise en décharge et le bio-engrais. Dans cette étude, on a étudié la faisabilité de l'ultrasonisation individuelle (UlS) et de l'oxydation de Fenton (FO) et des procédés de ferro-sonication (FS) combinés sur la dégradation de la chlortétracycline (CTC) dans les boues d'eaux usées. Les paramètres UlS tels que l'amplitude et le temps de sonication ont été optimisés par la méthodologie de la surface de réponse (RSM) pour une meilleure optimisation du processus FS. La génération de radicaux hydroxyles hautement réactifs dans les procédés FO et FS a été comparée pour évaluer l'efficacité de dégradation de la CTC. L'augmentation du rapport entre le peroxyde d'hydrogène et la concentration en fer a montré une augmentation de la dégradation de la CTC dans le procédé FO; Alors que dans FS, une augmentation de la concentration en fer n'a pas montré d'effet significatif ($p > 0,05$) sur la dégradation de la CTC dans les boues. La concentration de fer estimée dans les boues (115 mg/kg) était suffisante pour dégrader le CTC sans addition de fer externe. Le seul ajustement du pH des boues à 3 était suffisant pour générer des radicaux hydroxylés in situ en utilisant le fer qui est déjà présent dans les boues. Cette observation a été soutenue par l'estimation des radicaux hydroxyle avec ajustement du pH de l'eau à 3 et avec et sans addition de fer. Les conditions optimales de fonctionnement de l'UlS se sont révélées être de 60% d'amplitude pendant 106 minutes en utilisant RSM. Par rapport aux UlS et FO autonomes au ratio 1: 1, FS a montré une dégradation croissante de la CTC de 15% et 8%, respectivement. En outre, les UlS de boues ont augmenté l'activité œstrogénique 1,5 fois plus élevée par rapport à FO. Les échantillons traités par FS n'ont pas montré d'activité oestrogénique.

Mots clés: Chlortétracycline; Boues; Ultrasonication; Oxydation de Fenton; Ferro-sonication; Toxicity.

Abstract

Residual emerging contaminants in wastewater sludge remain an obstacle for its wide and safe applications such as landfilling and bio-fertilizer. In this study, the feasibility of individual ultrasonication (UlS) and Fenton oxidation (FO) and combined, Ferro-sonication processes (Kakavandi *et al.*) on the degradation of chlortetracycline (CTC) in wastewater sludge was investigated. UlS parameters such as amplitude and sonication time were optimized by response surface methodology (RSM) for further optimization of FS process. Generation of highly reactive hydroxyl radicals in FO and FS processes were compared to evaluate the degradation efficiency of CTC. Increasing in the ratio of hydrogen peroxide and iron concentration showed increased CTC degradation in FO process; whereas in FS, an increase in iron concentration did not show any significant effect ($p>0.05$) on CTC degradation in sludge. The estimated iron concentration in sludge (115 mg/kg) was enough to degrade CTC without the addition of external iron. The only adjustment of sludge pH to 3 was enough to generate *in-situ* hydroxyl radicals by utilizing iron which is already present in the sludge. This observation was further supported by hydroxyl radical estimation with adjustment of water pH to 3 and with and without the addition of iron. The optimum operating UlS conditions were found to be 60% amplitude for 106 min by using RSM. Compared to standalone UlS and FO at 1:1 ratio, FS showed 15% and 6% increased CTC degradation respectively. In addition, UlS of sludge increased estrogenic activity 1.5 times higher compared to FO. FS treated samples did not show any estrogenic activity.

Keywords: Chlortetracycline; Sludge; Ultrasonication; Fenton; Ferro-sonication; Toxicity.

Introduction

Tetracyclines (TCs) are the most commonly used antibiotics in veterinary life for therapeutic and prophylactic purposes. Up to 90% of TCs are excreted as parent compound after administration in all kinds of living systems (Agwuh *et al.*, 2006) via urine and feces. Consequently, these TCs have been detected in wastewater (0.3-62 µg/L; (Karthikeyan *et al.*, 2006, Puicharla *et al.*, 2014)), wastewater sludge (15 µg/kg-7.1 mg/kg, (Yuan *et al.*, 2014)) and in animal manure (91 mg/kg, (Martínez-Carballo *et al.*, 2007)) samples. Further usage of this wastewater and biosolids produced from animal manure and wastewater sludge (WWS) containing these residual TCs in irrigation of agricultural fields and as biofertilizer results in contamination of soil. Hence, the land application of biosolids will be the point source for the accumulation of TCs in soil and further leaching into water sources, which cause food chain contamination.

Currently abundance of TCs antibiotic resistance genes were identified in wastewater (Laht *et al.*, 2014) and also increased levels of TCs antibiotic resistance in the soil after using biosolids of animal manure and biosolids of wastewater treatment plant (WWTP) (Sengeløv *et al.*, 2003). Hence, waste management practices seems to be controlled by the accumulation, development and spread of resistance; which has become a major threat to human and environmental ecology owing to toxicity. Consequently, upgrading the existing regulations to improve the efficiency of WWTPs resulted in increased biosolids production (Bastian, 1997, Mohapatra *et al.*, 2011, Qiu *et al.*, 2013). Even, biosolids application in soils is ever increasing because of safe and efficient waste disposal and resourceful utilization of fertilizer value of biosolids (Osborne, 1996). So the adoption of advanced oxidation processes (AOPs) for removing residual contaminants including TCs in wastewater sludge (WWS) significantly represent a tool for the production of safe biosolids.

Chlortetracycline (CTC) is the former antibiotic of TCs family and most commonly used in veterinary infections. CTC form complexes with metals which decrease the solubility of CTC and also its strong adsorption to organic matrix directly or through metal bridging makes CTC high partitioning into the WWS instead of wastewater (Jezowska-Bojczuk *et al.*, 1993, Puicharla *et al.*, 2014, Vartanian *et al.*, 1998). Even lower biodegradation of CTC in sludge in conventional treatment processes, such as composting and stabilization (Shafrir *et al.*, 2012) makes CTC accumulation in biosolids. Furthermore, CTC is the most phototoxic compound among TCs and has showed chlorosis at 100 µg/L in *Lemna gibba* (Brain *et al.*, 2004). Considering these, it is necessary to mineralize CTC in WWS before using it in production of bio-fertilizer. Studies revealed that AOPs are efficient in degrading recalcitrant compounds in complex matrices, such as sludge and soil. Hence, it is necessary to evaluate AOPs to effectively remove organic pollutants including CTC from sludge before its final discharge into the environment.

WWS have high solids concentration and complex matrix; so that it is difficult to apply AOPs efficiently to mineralize toxic compounds including CTC. Mass transfer is the major limiting factor for application of ultraviolet irradiation, ozonation and Fenton oxidation (Meeting *et al.*) AOPs in WWS. Ultrasonication (Kapucu *et al.*) AOP can overcome this limitation by increasing the solubilization of sludge and this process has been reported to be more efficient in lesser time (Khanal *et al.*, 2007) compared to mechanical, thermal, chemical and biological methods to break down the sludge. But the production of hydroxyl radicals ($OH^•$) in UlS is not efficient as ozonation and FO processes for effective mineralization of toxic compounds. Hence, combined UlS AOP with ozonation or FO processes treatment might have the synergistic effect and overcome these problems (decreased mass transfer resistance and boost $OH^•$). Among AOPs, FO has more capability to destroy refractory organic pollutants in complex matrixes (Chen *et al.*, 1997) and studies proved that FO process efficiency was enhanced by sludge reduction and effectiveness in operating cost when coupled with UlS (Xu *et al.*, 2010). More importantly, accumulation of CTC in sludge by adsorption to solids directly or by metal bridging to perform sludge solubilization or disintegration to release bound CTC into free form for further effective degradation.

In this context, UlS AOP combined with FO process definitely will have a synergistic impact by solubilization of sludge solids and release and degradation of CTC. Further, the released CTC will be simultaneously degraded by FO process. Combined UlS and FO treatment may not be efficient in complete mineralization of CTC and also form different oxidative by-products as sludge contain diverse contaminants. In order to determine the safety of treatment and formed intermediate compounds, it is desirable to estimate the toxicity of sludge after treatment. The present work aims to evaluate the efficacy of combined UlS and FO process compared to individual treatments in degrading selected antibiotic CTC in WWS. In the first part of this work, optimization of UlS process parameters, such as time and ultrasonic frequency was performed using response surface methodology (RSM). And further combined UlS and FO that is ferro-sonication [2] process efficiency in degrading CTC was investigated by using optimized UlS parameters in WWS. Individual and combined treatment parameters are explored to determine the synergistic effect of FS on CTC degradation. In the second part, formed degradation products are analyzed and compared; further change in the toxicity of sludge following treatments is measured by using yeast estrogenicity screening (YES) assay and discussed along with degradation products.

Material and methods

Chemicals

Chlortetracycline hydrochloride (99% purity) was purchased from Toronto Research Chemicals (Toronto, Canada). Disodium ethylenediaminetetraacetate (Na_2H_2EDTA, 99%) was purchased from E-bay (Tokyo, Japan). Ammonium hydroxide (NH_4OH, 99.5%, 28-30% w/w) and methanol (HPLC grade, purity >99.8%) were purchased from Fisher Scientific (Ontario, Canada). The beef extract was purchased from Quelab Laboratories inc. (Montreal). Sodium dibasic phosphate (K_2HPO_4, 98%), sodium hydroxide (NaOH, 99.9%, 10N) and sodium monobasic phosphate (KH_2PO_4, 98%) was supplied by Laboratoire MAT Inc. (Quebec, Canada). Calcium chloride anhydrous ($CaCl_2$, >99%), magnesium perchlorate anhydrous ($Mg(ClO_4)_2$, >99%), cupric chloride anhydrous ($CuCl_2$, >99%), urea (>99%), iron sulfate heptahydrate ($FeSO_4.7H_2O$; 99% analytical grade), hydrogen peroxide (H_2O_2; 30%) and chromium nitrate ($(CrNO_3)_3.9H_2O$) were obtained from Fisher scientific (New Jersey, USA). pH of the WWS was adjusted by using H_2SO_4 (99.9%, 10N) or NaOH (5M) (Merck, US). N, N-Dimethyl-4-nitrosoaniline (RNO, 97%) was purchased from Sigma-Aldrich (Canada).

Milli-Q/Millli-Ro Milli pore system (Milford, MA, USA) was used to prepare HPLC grade water in the laboratory.

Wastewater sludge sampling and characterization

Wastewater sludge used in this study was collected from Quebec city WWTP (Sainte-Foy, Quebec, Canada) which has a sewage treatment capacity of 13140 m^3/h. Sludge samples were collected from sludge thickening source in WWTP and stored under dark conditions at 4 ± 1 ^oC to prevent degradation of CTC. Basic characterization of sludge, such as pH, total solids (TS), suspended solids [3], volatile solids (VS), soluble chemical oxygen demand (SCOD) was carried out as per the standard methods (Way, 2012). The specific characteristics of WWS used in this study are presented in table 4.1.1.

Experimental design and optimization by response surface methodology (RSM)

Experimental design and optimization of multivariable system was carried out by using RSM. In this study, initial optimization of independent variables (UlS time and amplitude) for optimal response of CTC degradation and its release into the supernatant was obtained by RSM. The responses are displayed in three-dimensional plots as a function of independent factors. Effect of independent variables: sonication time and amplitude of UlS was investigated for degradation in sludge and release of CTC into the supernatant as dependable variables using second order central composite design (CCD). CCD with a quadratic surface is usually appropriate for the process optimization. Independent variables and their experimental range are given in table 4.1.2.

Thirteen experiments were carried out according to the central composite to evaluate two UlS independent variables. The following quadratic (second-order) polynomial equation was used to fit the experimental results (see appendix, eq 1).

Where y is the predicted response, β_0 is the model intercept; β_i and β_{ij} are the coefficients of linear terms; β_{ii} is the coefficient of the quadratic term; X_i and X_j are the independent variables. *p*-value and F-value with 95% confidence level were determined from the analysis of variance (ANOVA). Each individual coefficient was determined by multiple linear regression and evaluation with 95% confidence level. The coefficient of determination (R^2) and the adjusted coefficient of determination (R^2adj) were used to express the extent of fitting of the experimental results to the polynomial model equation. Statistica 13 software (StatSoft base, USA) was used to perform all calculations and graphs of resulting data. Duplicate of 13 experiments and negative and positive controls were used to measure the degradation of CTC at room temperature.

In order to explore the synergistic effect of FS process in degrading CTC in sludge, simultaneous optimization of FS process was carried out. This study is based on the transformation of the predicted studied responses: CTC_{sludge}, $CTC_{supernatant}$, TDS and UlS energy to a dimensionless partial desirability function ($d_{CTCsludge,}$, $d_{CTC\ supernatant}$, d_{TDS} and d_{Energy}) (see appendix, eq.2-14). The objective is to minimize all the studied responses as given in the Equation 1 (see appendix eq.1).

Where A and B are the lowest and highest values observed in our experiments, respectively for all the studied responses. The global desirability function *D* is the combination of all partial desirability functions and consequently can be defined as:

Sonication experiments

Ultrasonic single frequency generator at 20 kHz (ultrasonic homogenizer Autotune 750W Cole-Parmer Instruments, Vernon Hills, Illinois, UlS) was used at different percentages of amplitude to optimize the power of UlS to disintegrate the sludge or degrade CTC in WWS. Sonication was carried out in continuous mode. Owing to the limitations of the equipment, amplitude below 20% and above 100% was not possible to use. Power dissipated into the WWS was displayed by the instrument in joules and ultrasonic intensity was calculated as per equation 15 (see appendix, eq.15). All the experiments were performed in 150 ml of WWS sample in 250 ml glass beaker with the ultrasonic probe (platinum) being immersed 2 cm into sludge.

Fenton oxidation

As reported in previous studies, the optimal dose of H_2O_2 and Fe^{2+} was between 25-60 mg and 1-50 mg per gram of TS of sludge, respectively and the molar ratio of H_2O_2 and Fe^{2+} ranged from 1:1 to 150: 1. In this study, the average of H_2O_2 (30 mg) and Fe^{2+} (30 mg) per gram of TS and molar ratio from 1:1 to 100:1 was considered. Thickened sludge used in this study has 34 g/L of TS; hence 0.45 mL of H_2O_2 (30% w/w) and H_2O_2

to Fe^{2+} ratio 1:1, 10:1, 25:1, 50:1 to 100: 1 was added in the form of $FeSO_4·7H_2O$ in series of experiments in 150 mL of sludge. WWS samples were acidified to pH 3.0±0.2 with 10N H_2SO_4 and were stirred uniformly at 150 rpm for 106 min. The Fenton reaction time was determined from optimized parameters of UlS. At the end of the experiment, 5N NaOH was used to quench the Fenton reaction by adjusting the pH 7.

Ferro-sonication

In the case of FS, WWS pH was adjusted to the desired value (pH 3) and the optimized UlS process conditions were used in FS experiments, such as 106 min and 60% amplitude. A similar molar ratio of Fe^{2+} of FO was added in FS experiments to study synergistic effect of combined FO and UlS.

Analytical methods

Hydroxyl radicals ($OH^•$) estimation:

The concentration of $OH^•$ was determined by ultraviolet (UV)-vis absorption spectroscopy using RNO which is an organic dye having a strong yellow color in aqueous solution. The selective bleaching of RNO by $OH^•$ and not reacting with other radicals, such as superoxide anions (O_2^-), singlet oxygen ($^1O_2$), or other peroxy compounds makes this compound specific for indirect estimation of $OH^•$ (Simonsen *et al.*, 2010). Absorbance measurements of RNO were performed at 440 nm (UV 0811 M136, Varian, Australia) as a function of RNO concentrations and it has maximum light absorption at neutral or basic pH. (Kraljić *et al.*, 1965)Kraljić et al, 1965 studies have demonstrated that RNO cannot be attacked by peroxy intermediates or peroxide anion in alkaline medium. After FO or FS experiments, pH was adjusted to 7 with NaOH and the resulting sample was centrifuged at 7650 × *g* to separate insoluble particles.

Chlortetracycline analysis

Preparation of samples

Solid fraction of treated sludge samples was separated by centrifugation at 6708 × *g* for 30 min. The resulting precipitate was freeze-dried (Dura Frezz Dryer, Kinetics). CTC extraction from lyophilized samples was carried out by microwave assisted extraction method (MARS microwave extractor, CEM Corporation, North Carolina, USA). About

0.5 grams of lyophilized sample was placed in the teflon tubes followed by addition of 20 mL of methanol. The extraction was performed in a temperature controlled mode and programmed as follows: temperature ramp to 100^{o}C for 10 min and holding at 100^{o}C for 15 min. This extraction system allows 14 vessels to be extracted simultaneously. This procedure was repeated twice for every sample and the extracts were combined, concentrated to 1 mL with the gentle stream of nitrogen evaporator and finally re-dissolved in 100 mL of milli-Q water.

Solid phase extraction

Solid phase extraction was employed to clean and concentrate the microwave extracted samples by using Sep-pack® C18 Plus Short Cartridges. Cartridges were fixed in a vacuum manifold (Welch, USA) connecting to a vacuum pump (Welch Rietschle Thomas, USA) for extracting the CTC from sludge sample. Initially, pH of WWS samples was adjusted to 3.5±0.5 with 2M acetic acid and 80 mg of Na_2H_2EDTA was added to the sample to make CTC free from metal complexation. Before sample loading, each cartridge was preconditioned with 5 mL methanol followed by water and 3.5±0.5 pH adjusted water. After conditioning, loaded samples were extracted at a flow rate of approximately 2 mL/min and then cartridges were dried using a vacuum system set at 15 psi for 15 min. Finally, CTC was eluted with 7 mL of methanol at a flow rate of 1 mL/min. Extract was dried and reconstituted with 1 mL of methanol before analysis using laser diode thermal desorption (LDTD) mass spectrometry (MS/MS) (Puicharla *et al.*, 2014).

LDTD-APCI-MS/MS analysis

Quantification of CTC concentration in WWS samples was done by LDTD-MS/MS (Phytronix technologies, Quebec, Canada) method developed by (Puicharla *et al.*, 2014) Pulicharla et al., [25]. LazWell TM plate (96-well plate) was used to introduce samples for analysis in LDTD-atmospheric chemical ionization (APCI) source. EDTA at 0.1 mg/mL was added to standard and samples for complete thermal desorption of CTC from the plate. Samples were spluttered (5 µL) onto the plate and left to dry at room temperature before introducing the plate into the APCI source. An infrared laser diode (980nm, 20W, continuous) was focalized on the back of the plate to thermally desorb sample from the plate. The desorbed sample was carried into APCI source by a stream of carrier gas (compressed air). The sample was ionized by APCI and analyzed by MS/MS. The internal standard method was used to quantify CTC and clomiphene citrate (10 µg/L) was used as internal standard. The area ratio of analyte : internal standard was plotted against the analyte concentration for the calibration curve and each sample was analyzed in 10 seconds.

Metal analysis-ICP-AES

Overall estimation of metal content in liquid and solid samples resulting from UlS treated samples was carried out by acid digestion using an autoclave to get a clear sample for ICP-AES metal analysis (Puicharla *et al.*, 2014). For liquid samples separated from sludge, concentrated HNO_3 was added for complete digestion. For lyophilized solid sample, along with concentrated HNO_3, $HClO_4$ and HF were also added; particularly to digest the silica present in the solid sample to get a clear solution. Acid digestion was carried out in a pressure (17 psi) controlled manner at 120 ±1 ^{0}C for 2 h.

Particle size analysis

Particle size analysis was carried out by a Horiba particle size 133 analyzer (LA-950 Laser Particle Size Analyzer, HORIBA, Edison, NJ, USA). During the entire analysis, moderate operating speeds of stirrer and recirculation pump were maintained to minimize the damage of sludge flocs. The sample was diluted 100 folds in tap water and analyzed in duplicate. Recorded readings were taken in 100 discrete particle ranges between 0.01 - 3000 µm as particle mean size and average values were taken and this method is based on the laser diffraction principles. The standard deviation of particle size measurement was below 13%.

Yeast estrogen screen (YES) assay:

The estrogenicity activity of resulting treated (FO and FS) WWS solution was evaluated by YES assay. This test was carried out based on the estrogen induced bioluminescence (Sanseverino *et al.*, 2009) assay which has similar detection limits to calorimetric based assay (Routledge *et al.*, 1996). All samples were filter sterilized prior to YES assay. The total estrogenic activity measurement was carried out by serial dilution method in a 96-well plate (Costar Brand, NY, USA) in luminescence spectrophotometer (Luminoskan™ Ascent Microplate Luminometer). Twenty four hours incubated yeast assay medium (hER-transfected recombinant yeast) diluted to 0.2 optical density was centrifuged at 7500 × g and supernatant was replaced with 250 µL of 4 times concentrated blank assay media and 750 µL of filter sterilized sludge and standard samples. These solutions were well mixed and incubated for 2 h at 30 ^{o}C. After incubation, 100 µL aliquots of each sample were transferred, in duplicate to a 96-well plate and 100 µL of Gal-screen (chemiluminescent substrate for luminometer) was added. Samples were sealed with parafilm and incubated for 2 h at 30 ^{o}C. After incubation, luminescence was measured at 420 nm.

Results and discussion

Preliminary screening - UlS

The preliminary screening experiments were done to optimize the independent variables of UlS parameters, such as sonication time (X_1, min) and amplitude (X_2, %). Amplitude is indirectly expressed as sonochemical efficiency (η) which is determined based on the production of $HO^{\cdot}$ radicals (Nowak, 2010). Studies of (Al-Juboori *et al.*, 2015)Juboori *et al*., 2015 calculated the sonication efficiency in terms of power consumption using following equation 16 (see appendix, eq. 16) .

Where (P_{Cal}) is calorimetric power (vibrational energy) and P_{Elec} is electrical power consumption which was imposed by the operator using an external waveform generator, P_{Cal} was calculated by using following equation (17) (see appendix, eq. 17).

In this expression, *(dT/dt)* represents the increase in the temperature of water ($^{o}C\ s^{-1}$), C_p is the calorific capacity of the liquid (4.2 $J.g^{-1}C^{-1}$), M is the sludge weight (g) that was subjected to treatment and t_{UlS} represents the sonication time (s).

In order to get the optimum values of independent variables, desirability function (*d*) was applied to the results of the dependable variables given in table 4.1.3 for the best agreement. As shown in figure 4.1.1, four dependable variables such as CTC in sludge, CTC in the supernatant, UlS energy and TDS were considered for obtaining the optimum values which are significantly ($p<0.05$) affected by treatment. The optimum UlS parameters were found to be 106 min sonication time and 60% amplitude. Figure 4.1.1 shows the effect of sonication time and amplitude on mentioned dependent variables individually. At these optimum conditions, 67% of CTC was degraded and its concentration in the supernatant was increased from below detection limit (BDL) to 17 mg/L. And also, other dependable variables such as 60% SCOD increment in sludge supernatant and reduction in floc size from 63 microns to 25 microns were obtained. Further, no significant (p>0.05) changes were observed in the pH and TVS of sludge over the experimental range. Figure 4.1.2 shows the combined effect of UlS time and amplitude on considered dependable variables using response surface plots.

Higher degradation of CTC in sludge was observed with increasing in amplitude and UlS intensity; which indicates higher energy is needed to degrade CTC in sludge. Increased TDS and SCOD in the supernatant were observed with increasing amplitude and sonication time due to higher solubilization of sludge solids which resulted in high release of CTC into supernatant. These results directly correlate the increased CTC in the supernatant to sludge solids solubilization or disintegration on which CTC is adsorbed (Kim *et al.*, 2005). Previous studies of (Wang *et al.*, 1999)Wang *et al*., 1999 have shown increased SCOD and TDS concentrations in UlS sludge compared to control. For comparing importance of sonication time and amplitude on each dependent variable, basic relationship between amplitude and energy (E) was taken and the equation is given as follows (see appendix, eq. 18).

Compared to sonication time, amplitude has major effect in degradation of CTC in sludge and its release into supernatant. As per equation 18, where doubling the amplitude transports four-fold of the vibrational energy by ultrasonic waves. Hence, high energy transfer resulted in higher degradation and disintegration of CTC and sludge flocks respectively. In case of TDS, sonication time has major impact compared to amplitude. This is because sludge solubilization or disintegration process needs time for organic matter (soluble protein and carbohydrate concentration) to transfer from sludge solid to liquid phase. Studies of (Kapucu *et al.*, 2000) Kapucu *et al*., 2000 proved that the increased release of protein from cell was the function of UlS time at constant power. SCOD increment in sludge supernatant and floc size reduction was dependent on both the treatment time and amplitude (Na *et al.*, 2007).

Further, significance of independent variables on different dependent variables was given in table 4.1.4. Change in the amplitude has more significant (*p*=0) effect on all dependable variables compared to sonication time (*p*<0.002). Higher degradation and release of CTC into sludge and sludge liquid at highest amplitude (99%, in trial 4) was due to enhanced production of hydroxyl radicals and elevated temperature during sonication (Yan *et al.*, 2010). Higher amplitude results in more acoustic cavitation in sludge which led to sludge floc disintegration and more solubilization of sludge. In trial 3, highest TDS was observed with 120 min sonication time and 88% amplitude. Hence, for solubilization of sludge, not only the energy, but longer sonication time is required (X_1X_2: $p<0.03$; table 4.1.4) which results in increased TDS and SCOD in sludge supernatant (figure 4.1.2). If trial 2 and 3 are compared (Table 4.1.3), both have same sonication time of 120 min with different amplitudes of 32 and 88, respectively. Trial 3 showed higher SCOD and CTC in supernatant compared to trial 2. Hence, longer sonication time at lower amplitude (lower energy) was unable to degrade or disintegrate the sludge which resulted in larger floc size, lower TDS and thus lesser CTC release into the supernatant (Kapucu *et al.*, 2000, Na *et al.*, 2007). Amplitude has high significant effect (*p*< 0.001; table 4.1.4) on CTC release in to the supernatant compared to sonication time (*p*<0.3; table 4.1.4). SCOD represents the amount of organic matter and reducing substances, such as nitrites, sulfides, amines and ferrous salts (Gong *et al.*, 2015). Therefore, ultrasonication with obtained optimum sonication time and amplitude is considered as suitable treatment to solubilize the sludge and further degrade the CTC and its release into the supernatant.

Correlating CTC release with metal concentration

Table 4.1.5 presents the different metal concentrations in various sludge supernatants after carrying out RSM of UlS treatment in WWS. These metals concentrations are correlated with CTC released into the supernatant. Calcium, magnesium, iron and aluminum concentration was significantly changed (p<0.001) in the supernatant after UlS treatment compared to raw sludge. Other metals, such as copper, zinc and cobalt did not show any significant (p>0.05) change in concentration after treatment. Metal ions which typically occur as mixture of metal oxides and are retained in the sludge solids due to their hydrophobic nature (Page *et al.*, 1981). Production of heat and hydroxyl radicals during ultrasonication oxidizes metal oxides along with organic matter which leads to release of soluble metal ions into the supernatant (Koenings, 1976, Taujale, 2015). Hence, there was a clear correlation with the increase in the metals concentration, such as calcium, magnesium and iron in the supernatant with increase in CTC concentration. Studies confirmed that CTC was efficient in complexing with Ca^{2+} and Mg^{2+} and also adsorbing onto sludge organic matrix via metal bridging (Carlotti *et al.*, 2012, Pulicharla *et al.*, 2015). The breakdown of sludge floc might have cleaved the CTC metal bridging to organic matter (Pils *et al.*, 2007) or CTC metal complexes and released it into the supernatant as free CTC, CTC-metal complex or CTC metal complex adsorbed to dissolved organic matter. It seems that CTC was released into sludge supernatant along with metals as CTC metal complexes. Further studies are needed to confirm the effect of metal ions on release of CTC into supernatant during UlS.

CTC degradation

Comparison among UlS, FO and FS

CTC degradation efficiency of UlS, FO and FS is shown in figure 4.1.3. As shown in figure 4.1.3, CTC degradation in sludge decreased with increase in the ratio of H_2O_2 and $FeSO_4.7H_2O$ and there was no significant difference ($p>0.05$) in the degradation of CTC over the ratio of Fe^{2+} addition into the sludge in the case of FS. Optimum conditions of the RSM design have shown 67% degradation of CTC and authors used these optimum conditions for combined FS process. For all experiments of FS, the UlS parameters were alike and only the iron concentration was changed from 0.3-30 mg of Fe^{+2}/g of TS. Hence, there was no change in the UlS energy, time of sonication and composition of sludge, such as TDS, TS, and SCOD during all FS experiments. The change in energy was same for Fenton experiments and only the ratio of H_2O_2 and Fe^{2+} was varied. CTC degradation of 67%, 76% and 82% was observed for UlS, FO (1:1) and FS, respectively. Combined treatment FS showed 15% and 8% increment in the degradation of CTC compared to standalone UlS and FO, respectively. In FO process, the oxidant H_2O_2 concentration significantly influences the degradation of CTC. According to the results, rise in ratio of H_2O_2:Fe^{2+} enhanced the CTC degradation. And maximum degradation was achieved at 1:1 ratio. Similar results were shown in the previous studies where high OH$^{\bullet}$ was produced due to high decomposition of H_2O_2 (Kakavandi *et al.*, 2016).

In previous study of Hou *et al*. 2016 (Hou *et al.*, 2016), less than 10% of tetracycline removal was obtained in water when UlS and H_2O_2 were employed separately. In this study, FS experiment without addition of iron showed similar CTC degradation (80-82%) where FO experiment without addition of iron resulted in variable degradation of CTC from 35-40%. Sludge which already contains high concentration of iron might be utilized in the individual treatment and results in high degradation of CTC. Comparing to FS, Fenton reagent failed to effectively use the iron which was present in the sludge to generate hydroxyl radicals. In FO treatment, there is no source to convert the insoluble iron oxide into soluble form for further utilization to produce OH$^{\bullet}$. In contrast, for FS, energy input by UlS seemed to be enough to degrade CTC by increasing the utilizing of iron present in the sludge. And also solubilization of sludge release the adsorbed CTC into supernatant and increase the overall availability of CTC to OH$^{\bullet}$ radicals resulting in higher degradation (Mohapatra *et al.*, 2011). This can be further supported by the increment of Fe^{2+} concentration in the supernatant after UlS (Table 4.1.5) which helped in the generation of hydroxyl radicals (Koenings, 1976). Not only the hydroxide radical generation, but also the additional formation of various inorganic radicals, such as SO4$^{\cdot-}$, Cl$^{\cdot}$, $Cl_2^{\cdot-}$ might contribute to increased CTC degradation efficiency in case of FS (Siedlecka *et al.*, 2005). Therefore, FS treatment with optimized parameters was considered as an advanced combined AOP that played a vital role in simultaneous degradation and its release of CTC into sludge supernatant. Simple adjustment of sludge pH 3 was enough without utilization of external iron for generation of OH$^{\bullet}$ radicals in this process.

Correlation with hydroxyl radicals

Figure 4.1.3 represents the comparison of hydroxyl radical production with degradation of CTC. The increase in generation of OH radicals was observed in case of FO experiments (Mohapatra *et al.*, 2011) which were in concordance with the degradation of CTC. This explained the fact that Fenton reagent was unable to utilize the iron present in the sludge and it needed the external addition of Fe^{2+} for the production of OH radicals to degrade CTC. Unlike FO, concordance in the generation of OH radicals and degradation of CTC in sludge was not found for FS experiments. This might be due to the presence of higher concentration of iron in the sludge (115 mg/kg) and further addition of iron did not increase the production of hydroxyl radicals. In FS, sonication is efficient in solubilization of sludge and releases the ferrous salts into supernatant (Gong *et al.*, 2015) which is supported by the reduction in sludge floc size, increment in TDS and increase in iron concentration in the supernatant. Hence, simple adjustment of sludge pH to 3 in FS led to generation of OH radical and simultaneous degradation of free or metal-complexed CTC confirming combined process FS as having synergistic effect. The synergistic effect in degradation of CTC found in this study was comparable with previous studies where above 90% degradation of tetracycline was found in municipal wastewater (Wang *et al.*, 1999).

Lower efficiency of CTC degradation in sludge compared to WW effluent was due to presence of higher solids which consumes part of energy to solubilize and thus prevents complete energy transfer to degrade CTC (Le *et al.*, 2013). Further CTC metal complexation and its adsorption to organic matrix might decrease the CTC degradation efficiency. Studies of (Zhao *et al.*, 2015) Zhao *et al*., 2015 proved the presence of metals affects the degradation efficiency of tetracycline. Accelerated and reduced degradation of tetracycline in the presence of low and high concentration of metals respectively was observed. Hence, CTC degradation in sludge might show lower efficiency due to higher solids and higher metals concentration which had negative effects on degradation of pollutants. As sludge is a complex matrix having diverse metals further studies to specify the effect of metals on degradation of CTC in real sludge conditions are necessary.

Effect of variable on CTC degradation

In this study, UlS, FO and FS processes did not achieve 100% degradation of CTC. Previous studies have shown above 90% degradation of CTC in spiked surface and drinking water (Daghrir *et al.*, 2014). Although enough OH radicals were generated in sludge as it contains high Fe^{2+} metal ions, still they might not be efficient in degrading

CTC due to presence of higher TS which caused scavenging of generated OH radicals (Zepp *et al.*, 1992). Generation of 30% OH radicals in the ultrasonicated synthetic sludge at pH 3 was seen without the addition of iron compare to water (Mowla *et al.*, 2014). This result indicates that other than Fe^{2+} ions, organic compounds such as nitrates and dissolved organic matter at high temperature are also involved in generation of OH radicals (Dong *et al.*, 2012, McKay *et al.*, 2015). Considering the physicochemical properties of CTC, such as higher adsorption and metal complexation, this aided in CTC retention in sludge and also affected negatively in the degradation of CTC (Pils *et al.*, 2007). Figure 4.1.4 shows the significance of TS on degradation of CTC compared to metal concentration. As shown in figure 4.1.4, metal concentration in sludge solids and supernatant of UlS and FS treated sludge samples was almost similar, but 43% and 84% degradation efficiency was seen in sludge and supernatant of FS samples, respectively by comparing CTC in UlS sludge and supernatant as a reference. CTC which was released into sludge supernatant was prone to degradation compared to CTC retained in the sludge even though CTC was in metal-complexed form in the supernatant. Hence, compared to metal complexation, total solids seemed to be having a relatively higher negative effect on degradation of CTC (Rivera-Utrilla *et al.*, 2013). Overall, the degradation process of CTC was a combination of various factors: 1) the presence of complex matrix; 2) physical-chemical properties of degrading compound and; 3) interaction among matrix compounds.

By-products (BPs) formation

Incomplete mineralization of CTC during FO and FS treatments resulted in the formation of intermediate BPs which may pose risks to the environment. LDTD-MS/MS spectra obtained before and after degradation were compared to identify the formed intermediates (m/z: $[M+H]^+/z$) and the degradation pathways for CTC. Several BPs were identified in FS (10 BPs) compared to FO (6 BPs) treatment. Intermediate products, such as *m/z* 415 and 449 were identified in case of FS which was comparable to previous studies (Khan *et al.*, 2016). Figure 4.1.4 illustrates the degradation pathway and BPs of CTC detected after FO (Figure 4.1.4 (A)) and FS (Figure 4.1.4 (B)) treatments. In this study, *m/z* having low mass intermediates, such as 358.7, 330.7, 284.7, 152, 144.5, 130.5 in case of FO and 392.8, 320.7, 244.6, 234.7, 216.7, 186.6, 144.6, 110 in case of FS were reported for the first time. Degradation pathways were proposed based on this BPs (Figure 4.1.4) that suggested the major difference in the degradation pathways of both treatments. Conclusion on the degradation mechanisms of CTC cannot be done based on these tentative structures and need further studies such as Nuclear magnetic resonance spectroscopy (NMR) to confirm the degradation mechanism.

In both FO and FS, hydroxyl radicals are prone to attack C4-dimethyl amino and C2-carboxyl amide groups of CTC (Khan *et al.*, 2016). And further oxidation by OH radicals resulted in the formation of tricyclic and bicyclic aromatic intermediate compounds. These intermediates undergo ring cleavage and are subsequently mineralized into carbon dioxide and water. OH radicals involve in a non-selective attack on cleavage of functional groups and hydroxylation of the aromatic rings of CTC leading to smaller organic fragments. The difference in BPs in both FO and FS might be due to difference in the quantity of OH radicals produced and also the input of UlS energy in case of FS. No literature was found to correlate the formed BPs and its degradation mechanism particularly at low mass BPs.

Estrogenicity assessment of UlS, FO and FS treated wastewater sludge samples

Estrogen activity of sludge supernatant before and after treatment was carried out by YES test. Before starting the YES test, supernatant of sludge samples (raw sludge, UlS, FO, and FS) were filtered sterilized to effectively remove the microorganisms. Genetically engineered yeast having a promoter carrying human estrogen receptor which can express estrogen-responsive sequences once activated in an estrogen dependent manner was used in the YES assay (Sanseverino *et al.*, 2009). Sensitivity and reproducibility of this assay were assessed by measuring luminescence of yeast cells exposed to standard 17β-estradiol solution and β-Galactosidase enzyme (Routledge *et al.*, 1996). Results showed that FS treated sludge samples showed estrogenic activity below detection limit of method (<1 ng/L) and it was with in the acceptable limit as per WHO/FAO (Meeting *et al.*, 2010). UlS treated samples have shown 1.5 times estrogenic activity that is approximately equal to 0.35μg/L of 17β-estradiol compared to FO samples having 0.2μg/L 17β-estradiol estrogen activity. This high estrogenic activity of UlS sludge samples explains that UlS treatment seems to be more efficient in solublization and release of toxic compound from sludge solids to liquid instead of their degradation. In FO process, generated OH radicals might be insufficient to degrade the toxic compounds in the sludge or the formed intermediate BPs in FO process might have estrogenic activity compared to BPs of FS. Hence, FS sludge treatment was pertinent for safe disposal of WWS; however further toxicity testing of treated sludge samples at different time points should be done to obtain detailed toxicity information of formed byproducts which would indicate the further continuation of treatment duration, if required.

Conclusions

Ultrasonic degradation of CTC was studied using CCD and desirability functions were found to be 106 min and 60% amplitude. This optimization was performed to obtain the simultaneous degradation of CTC in sludge and release of CTC into the supernatant. Lower degradation efficiency (67%) of UlS was due to the lower generation of OH radicals, where FS showed 82% efficiency due to the utilization of iron to generate more OH radicals in sludge. UlS has the efficiency to cleave the linkage amongst CTC, metals and organic matrix and release into sludge supernatant. Increased metal concentrations (Ca, Mg, Al, Co, Cu and Fe) in supernatant along with CTC were responsible for breakdown of sludge flocs by the UlS. The release of CTC by UlS and utilization of iron at pH 3 (Meeting *et al.*) were main factors showing synergistic effect in CTC degradation in FS treatment. 1:1 ratio of FO process showed 76% degradation of CTC and whereas FS showed 82% of CTC in WWS. FO process was inefficient in utilizing the iron present in the sludge due to higher TS of sludge. Different BPs in both FO and FS might be due to variability in the quantity of OH radicals produced and also degradation by additional inorganic radicals, such as $SO^{4\cdot -}$, $Cl^{\cdot}$, $Cl_2^{\cdot -}$ generated by UlS. YES assay results showed that FS treated WWS samples were not estrogenic, confirming its safety for use in wastewater treatment plants.

Acknowledgement

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Discovery Grant 355254 and Strategic Grant), and Ministère des Relations internationales du Québec (coopération Québec-Catalanya 2012-2014) for financial support. Authors are also thankful to “merit scholarship program for foreign students” (FQRNT) for financial assistance to Rama Pulicharla. The views or opinions expressed in this article are those of the authors.

References

Agwuh KN & MacGowan A (2006) Pharmacokinetics and pharmacodynamics of the tetracyclines including glycylcyclines. *Journal of Antimicrobial Chemotherapy* 58(2):256-265.

Al-Juboori RA, Yusaf T & Bowtell L (2015) Energy conversion efficiency of pulsed ultrasound. *Energy Procedia* 75:1560-1568.

Bastian RK (1997) The biosolids (sludge) treatment, beneficial use, and disposal situation in the USA. *European Water Pollution Control* 2(7):62-79.

Brain RA, Johnson DJ, Richards SM, Sanderson H, Sibley PK & Solomon KR (2004) Effects of 25 pharmaceutical compounds to Lemna gibba using a seven-day static-renewal test. *Environmental Toxicology and Chemistry* 23(2):371-382.

Carlotti B, Cesaretti A & Elisei F (2012) Complexes of tetracyclines with divalent metal cations investigated by stationary and femtosecond-pulsed techniques. *Physical Chemistry Chemical Physics* 14(2):823-834.

Chen R & Pignatello JJ (1997) Role of quinone intermediates as electron shuttles in Fenton and photoassisted Fenton oxidations of aromatic compounds. *Environmental Science & Technology* 31(8):2399-2406.

Daghrir R, Drogui P, Delegan N & El Khakani MA (2014) Removal of chlortetracycline from spiked municipal wastewater using a photoelectrocatalytic process operated under sunlight irradiations. *Science of The Total Environment* 466:300-305.

Dong MM & Rosario-Ortiz FL (2012) Photochemical formation of hydroxyl radical from effluent organic matter. *Environmental science & technology* 46(7):3788-3794.

Gong C, Jiang J, Li Da & Tian S (2015) Ultrasonic application to boost hydroxyl radical formation during Fenton oxidation and release organic matter from sludge. *Scientific reports* 5.

Hou L, Wang L, Royer S & Zhang H (2016) Ultrasound-assisted heterogeneous Fenton-like degradation of tetracycline over a magnetite catalyst. *Journal of hazardous materials* 302:458-467.

Jezowska-Bojczuk M, Lambs L, Kozlowski H & Berthon G (1993) Metal ion-tetracycline interactions in biological fluids. 10. Structural investigations on copper (II) complexes of tetracycline, oxytetracycline, chlortetracycline, 4-(dedimethylamino) tetracycline, and 6-desoxy-6-demethyltetracycline and discussion of their binding modes. *Inorganic Chemistry* 32(4):428-437.

Kakavandi B, Takdastan A, Jaafarzadeh N, Azizi M, Mirzaei A & Azari A (2016) Application of Fe 3 O 4@ C catalyzing heterogeneous UV-Fenton system for tetracycline removal with a focus on optimization by a response surface method. *Journal of Photochemistry and Photobiology A: Chemistry* 314:178-188.

Kapucu H, Gülsoy N & Mehmetoğlu Ü (2000) Disruption and protein release kinetics by ultrasonication of Acetobacter peroxydans cells. *Biochemical engineering journal* 5(1):57-62.

Karthikeyan K & Meyer MT (2006) Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Science of the Total Environment* 361(1):196-207.

Khan MH & Jung J-Y (2016) Ozonation of chlortetracycline in the aqueous phase: Degradation intermediates and pathway confirmed by NMR. *Chemosphere* 152:31-38.

Khanal SK, Grewell D, Sung S & Van Leeuwen J (2007) Ultrasound applications in wastewater sludge pretreatment: a review. *Critical Reviews in Environmental Science and Technology* 37(4):277-313.

Kim S, Eichhorn P, Jensen JN, Weber AS & Aga DS (2005) Removal of antibiotics in wastewater: effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. *Environmental Science & Technology* 39(15):5816-5823.

Koenings JP (1976) In situ experiments on the dissolved and colloidal state of iron in an acid bog lake. *Limnology and Oceanography* 21(5):674-683.

Kraljić I & Trumbore C (1965) p-Nitrosodimethylaniline as an OH Radical Scavenger in Radiation Chemistry1. *Journal of the American Chemical Society* 87(12):2547-2550.

Laht M, Karkman A, Voolaid V, Ritz C, Tenson T, Virta M & Kisand V (2014) Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load. *PloS one* 9(8):e103705.

Le NT, Julcour-Lebigue C & Delmas H (2013) Ultrasonic sludge pretreatment under pressure. *Ultrasonics sonochemistry* 20(5):1203-1210.

Martínez-Carballo E, González-Barreiro C, Scharf S & Gans O (2007) Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. *Environmental Pollution* 148(2):570-579.

McKay G & Rosario-Ortiz FL (2015) Temperature dependence of the photochemical formation of hydroxyl radical from dissolved organic matter. *Environmental science & technology* 49(7):4147-4154.

Meeting JFWECoFA & Organization WH (2010) *Evaluation of Certain Food Additives: Seventy-first Report of the Joint FAO/WHO Expert Committee on Food Additives.* World Health Organization,

Mohapatra D, Brar S, Tyagi R & Surampalli R (2011) Parameter optimization of ferro-sonication pre-treatment process for degradation of bisphenol A and biodegradation from wastewater sludge using response surface model. *Journal of hazardous materials* 189(1):100-107.

Mowla A, Mehrvar M & Dhib R (2014) Combination of sonophotolysis and aerobic activated sludge processes for treatment of synthetic pharmaceutical wastewater. *Chemical Engineering Journal* 255:411-423.

Na S, Kim Y-U & Khim J (2007) Physiochemical properties of digested sewage sludge with ultrasonic treatment. *Ultrasonics sonochemistry* 14(3):281-285.

Nowak FM (2010) *Sonochemistry: Theory, Reactions, Syntheses, and Applications.* Nova Science Publishers,

Osborne G (1996) Fertilizer value of biosolids-a review. *Sydney: NSW Agriculture Organic Waste Recycling Unit and Sydney Water*.

Page A, Chang A, Sposito G & Mattigod S (1981) Trace elements in wastewater: their effects on plant growth and composition and their behavior in soils. *Modelling wastewater renovation, land treatment. JohnWiley & Sons, New York*:182-222.

Pils JR & Laird DA (2007) Sorption of tetracycline and chlortetracycline on K-and Ca-saturated soil clays, humic substances, and clay-humic complexes. *Environmental science & technology* 41(6):1928-1933.

Puicharla R, Mohapatra D, Brar S, Drogui P, Auger S & Surampalli R (2014) A persistent antibiotic partitioning and co-relation with metals in wastewater treatment plant—Chlortetracycline. *Journal of Environmental Chemical Engineering* 2(3):1596-1603.

Pulicharla R, Das RK, Brar SK, Drogui P, Sarma SJ, Verma M, Surampalli RY & Valero JR (2015) Toxicity of chlortetracycline and its metal complexes to model microorganisms in wastewater sludge. *Science of The Total Environment* 532:669-675.

Qiu S, Xia M & Li Z (2013) Ultrasonic irradiation as pretreatment for the reduction of excess sludge by Fenton-acclimation treatment. *Water Science & Technology* 67(8):1701-1707.

Rivera-Utrilla J, Gómez-Pacheco CV, Sánchez-Polo M, López-Peñalver JJ & Ocampo-Pérez R (2013) Tetracycline removal from water by adsorption/bioadsorption on activated carbons and sludge-derived adsorbents. *Journal of environmental management* 131:16-24.

Routledge EJ & Sumpter JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental toxicology and chemistry* 15(3):241-248.

Sanseverino J, Eldridge ML, Layton AC, Easter JP, Yarbrough J, Schultz TW & Sayler GS (2009) Screening of potentially hormonally active chemicals using bioluminescent yeast bioreporters. *Toxicological sciences* 107(1):122-134.

Sengeløv G, Agersø Y, Halling-Sørensen B, Baloda SB, Andersen JS & Jensen LB (2003) Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environment international* 28(7):587-595.

Shafrir M & Avisar D (2012) Development method for extracting and analyzing antibiotic and hormone residues from treated wastewater sludge and composted biosolids. *Water, Air, & Soil Pollution* 223(5):2571-2587.

Siedlecka E & Stepnowski P (2005) Phenols degradation by Fenton reaction in the presence of chlorides and sulfates. *Polish Journal of Environmental Studies* 14(6):823-828.

Simonsen ME, Muff J, Bennedsen LR, Kowalski KP & Søgaard EG (2010) Photocatalytic bleaching of p-nitrosodimethylaniline and a comparison to the performance of other AOP technologies. *Journal of Photochemistry and Photobiology A: Chemistry* 216(2):244-249.

Taujale S (2015) *Interactions between metal oxides and/or natural organic matter and their influence on the oxidative reactivity of manganese dioxide*. (TEMPLE UNIVERSITY).

Vartanian VH, Goolsby B & Brodbelt JS (1998) Identification of tetracycline antibiotics by electrospray ionization in a quadrupole ion trap. *Journal of the American Society for Mass Spectrometry* 9(10):1089-1098.

Wang Q, Kuninobu M, Kakimoto K, Hiroaki I & Kato Y (1999) Upgrading of anaerobic digestion of waste activated sludge by ultrasonic pretreatment. *Bioresource Technology* 68(3):309-313.

Way C (2012) Standard methods for the examination of water and wastewater. *Water Environment Federation, Secaucus, NJ, USA*.

Xu G, Chen S, Shi J, Wang S & Zhu G (2010) Combination treatment of ultrasound and ozone for improving solubilization and anaerobic biodegradability of waste activated sludge. *Journal of Hazardous Materials* 180(1):340-346.

Yan Y, Feng L, Zhang C, Zhu H & Zhou Q (2010) Effect of ultrasonic specific energy on waste activated sludge solubilization and enzyme activity. *African Journal of Biotechnology* 9(12).

Yuan X, Qiang Z, Ben W, Zhu B & Liu J (2014) Rapid detection of multiple class pharmaceuticals in both municipal wastewater and sludge with ultra high performance liquid chromatography tandem mass spectrometry. *Journal of Environmental Sciences* 26(9):1949-1959.

Zepp RG, Faust BC & Hoigne J (1992) Hydroxyl radical formation in aqueous reactions (pH 3-8) of iron (II) with hydrogen peroxide: the photo-Fenton reaction. *Environmental Science & Technology* 26(2):313-319.

Zhao B, Wang F, Bao J & Duan L (2015) Influence of Cd2+ and Pb2+ on ultrasonic degradation of tetracycline. *Chinese Journal of Environmental Engineering* 9(4):1599-1605.

Table 4.1.1 Physico-chemical characteristics of wastewater sludge

Parameter	**value**
pH	5.7±0.6
Total solids (g/L)	34.57±1.2
Suspended solids (g/L)	31.73±3.6
Total dissolved solids (g/L)	2.84±2.9
Volatile solids (g/L)	20.9±4.2
Total dissolved volatile solids (g/L)	1.22±5.1
Chemical oxygen demand (mg/L)	6558±8.3
CTC in supernatant (mg/L)	BDL
CTC in sludge (mg/kg)	34.22±2.7

CTC: chlortetracycline

Table 4.1.2 Experimental range and independent variables levels of response surface methodology

Factor	**Variable**	**Coded (levels)**				
		-1.41	-1	0	1	1.41
X_1	Sonication time [4]	11.36	30	75	120	138.64
X_2	Amplitude (%)	20.4	32	60	88	99.59

Table 4.1.3 Experimental results of central composite design and dependable variables affecting the degradation of CTC (n=3, SD<10%)

Trial no	**Sonication time (min)**	**Amplitude (%)**	**CTC in supernatant (mg/L)**	**TDS (mg/L)**	**CTC in sludge (mg/kg)**	**Ultrasonic intensity (Watt/cm^2)**	**Particle size (microns)**	**SCOD (mg/L)**
1	**138.64**	**60**	14.25	6.12	10.7	21.94	10.9	11966
2	**120**	**88**	16.12	10.15	11.18	40.58	10.31	11114
3	**120**	**32**	BDL	4.33	9.28	9.70	12.4	9884
4	**75**	**99**	49.67	7.19	8.45	49.51	11.63	11195
5	**75**	**60**	14.89	4.80	8.92	23.21	22.71	10982
6	**75**	**60**	15.75	5.20	9.22	22.92	23.33	10693
7	**75**	**60**	14.92	5.1	8.99	23.11	22.53	10991
8	**75**	**60**	14.22	4.9	9.02	22.83	21.23	10001
9	**75**	**60**	14.14	5.59	9.08	22.83	20.79	9943
10	**75**	**20**	5.01	3.7	9.3	5.86	14.87	7896
11	**30**	**88**	13.15	4.88	10.36	44.88	15.14	12899
12	**30**	**32**	BDL	3.23	11.4	12.43	53.57	9510
13	**11.36**	**60**	BDL	4.15	8.89	36.11	48.9	9753

CTC: Chlortetracycline; TDS: Total dissolved solids; SCOD: soluble chemical oxygen demand

Table 4.1.4 Significance (probability (*p*)) of independent variables on dependent variables

Model	CTC_{sludge}		$CTC_{supernatant}$		TDS		UIS energy	
	p	Effect	*p*	Effect	*p*	Effect	*p*	Effect
X_1	0.04359	-2.77894	0.24183	7.1426	0.0467	2.2890	0.00168	-6.81537
X_1^2	0.13796	2.03006	0.00792	-22.0145	0.83821	0.21575	0.00814	5.40536
X_2	0.36634	-1.09186	0.00011	43.3584	0.84595	0.1914	0.00000	31.26046
X_2^2	0.23937	1.55905	0.04808	14.3263	0.62147	0.52575	0.03044	4.00101
X_1X_2	0.161	2.50614	0.26916	9.4813	0.02722	-3.7350	0.69992	-0.78361

CTC: Chlortetracycline; TDS: Total dissolved solids; UIS: Ultrasonication

Significant values ($p < 0.05$)

Table 4.1.5 Metals and CTC concentration (mg/L) in sludge supernatant after ultrasonic treatment of sludge (n=3 and SD<10%)

Trial No	Sonication time (min) (X_1)	Amplitude (%) (X_2)	CTC	Ca 315.887	Mg 280.270	Fe 261.187	Al 396.152	Cu 327.395	Co 228.615	Zn 206.20
Raw sludge	0.0	0.0	BDL	743.56	52.48	9.33	1.46	0.08	0.021	0.21
1	138.64	60	4.25	604.29	51.81	9.65	1.93	0.09	0.025	0.14
2	120	88	16.12	1395.62	115.55	10.51	2.19	0.07	0.047	0.14
3	120	32	BDL	790.08	72.88	11.95	3.91	0.08	0.025	0.14
4	75	99	49.67	1216.41	101.65	10.26	3.59	0.09	0.037	0.24
5	75	20	5.01	741.25	68.54	12.29	4.59	0.08	0.018	0.29
6	75	60	14.22	842.42	77.12	8.42	2.31	0.06	0.03	0.17
7	75	60	14.14	890.32	75.32	12.42	2.34	0.07	0.03	0.15
8	75	60	14.89	857.87	76.68	9.61	2.42	0. 06	0.029	0.15
9	75	60	15.75	830.95	73.97	9.85	2.09	0.07	0.031	0.21
10	75	60	14.92	900.21	78.96	10.47	2.24	0.06	0.035	0.17
11	30	88	13.15	782.99	70.51	9.53	1.5	0.04	0.029	0.18
12	30	32	BDL	741.04	68.76	9.40	3.66	0.03	0.02	0.25
13	11.36	60	BDL	472.97	45.29	8.86	1.78	0.05	0.018	0.15

CTC: chlortetracycline; Ca: Calcium; Mg: magnesium; Cu: Cupper; Fe: Iron: Al: Aluminum; Co: Cobalt; Zn: Zinc

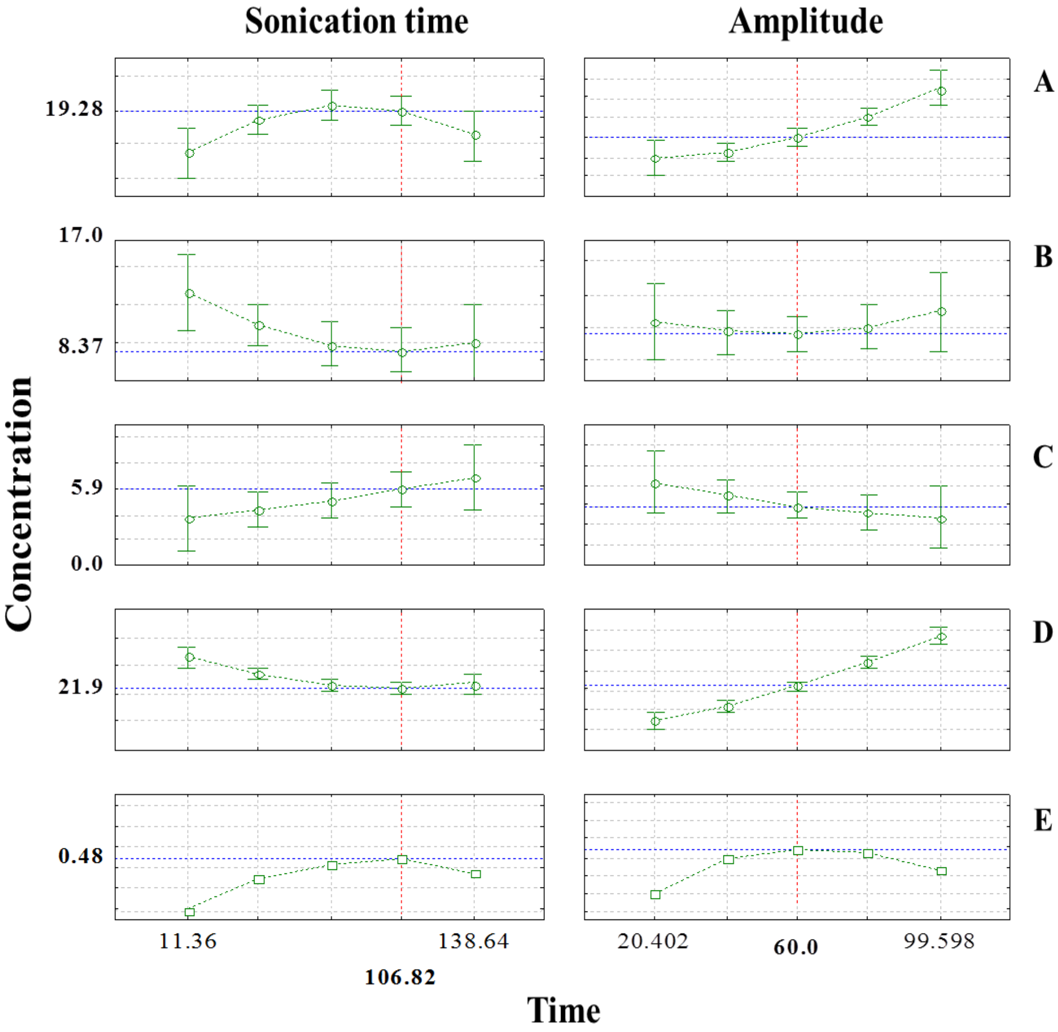


Figure 4.1.1 Predicted profiles of dependent variables and desirability plot of central composite design; A) CTC in supernatant; B) CTC in sludge; C) total dissolved solids; D) sonication energy; E) desirability values.

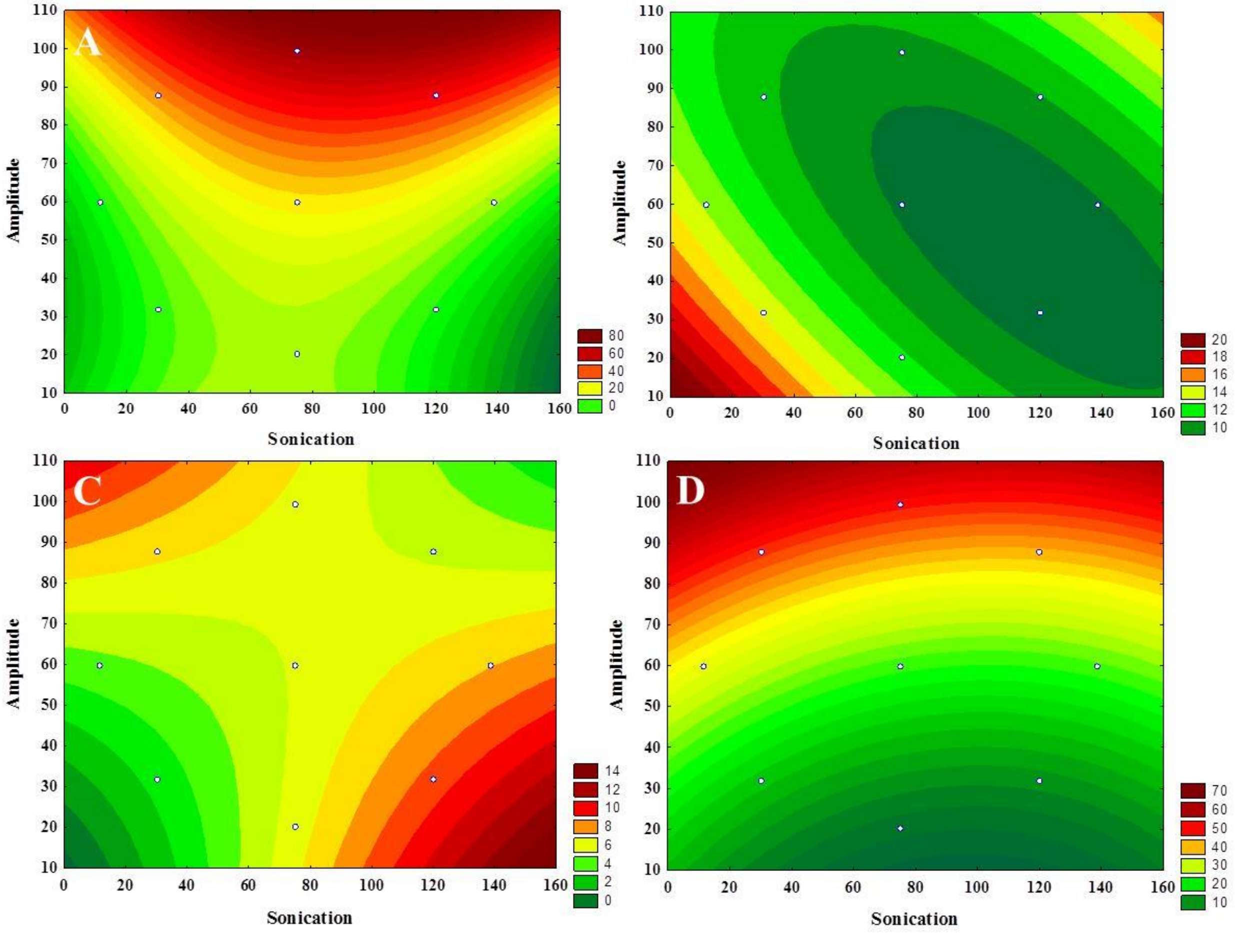


Figure 4.1.2 **Response surface plots as a function of sonication time and amplitude; A) CTC in supernatant (mg/L); B) CTC in sludge (mg/kg); C) total dissolved solids (mg/L); D) sonication energy (Watt/cm^2).**

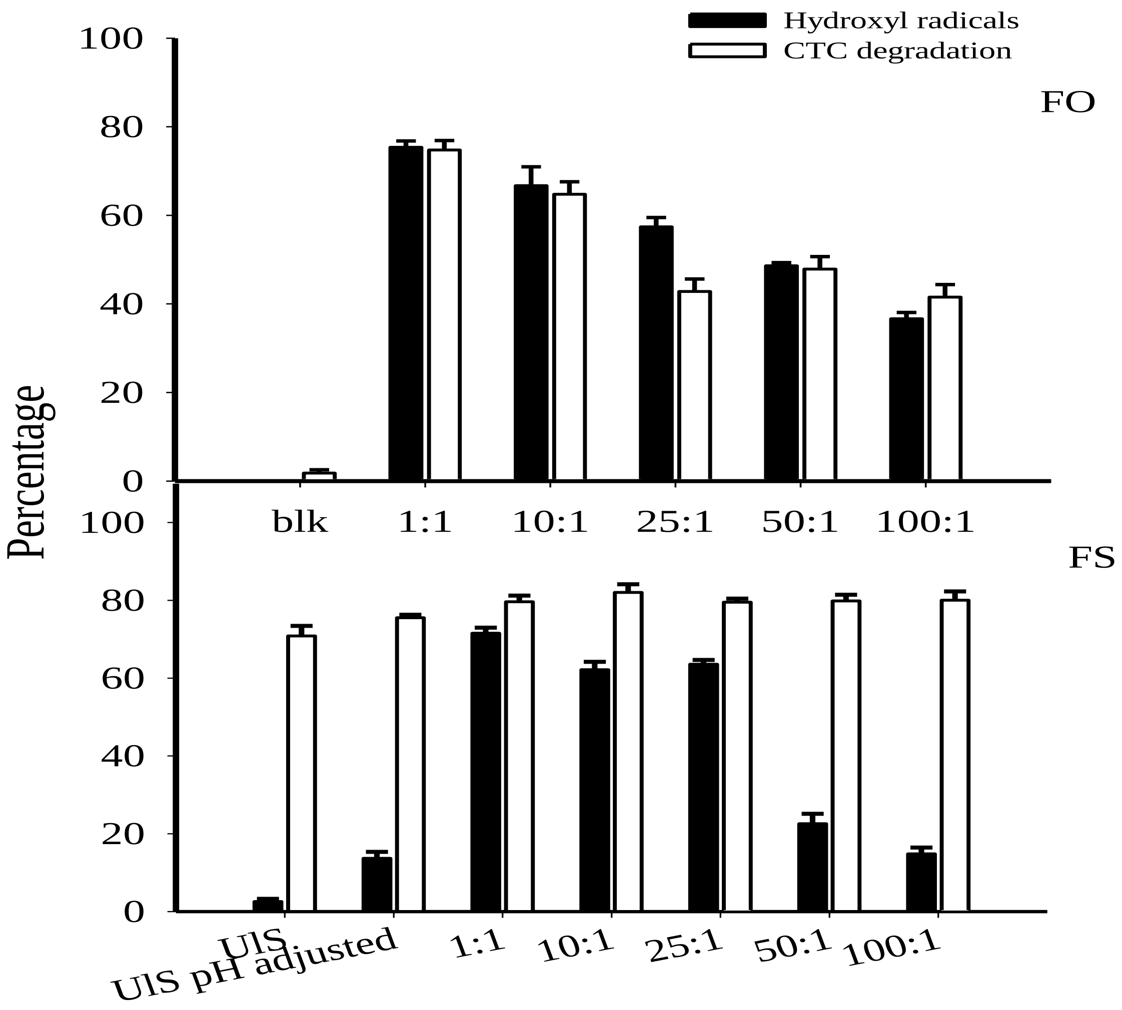


Figure 4.1.3 **Comparison of CTC degradation with production of hydroxyl radicals in Fenton oxidation (FO) and Ferro-sonication (FS)**

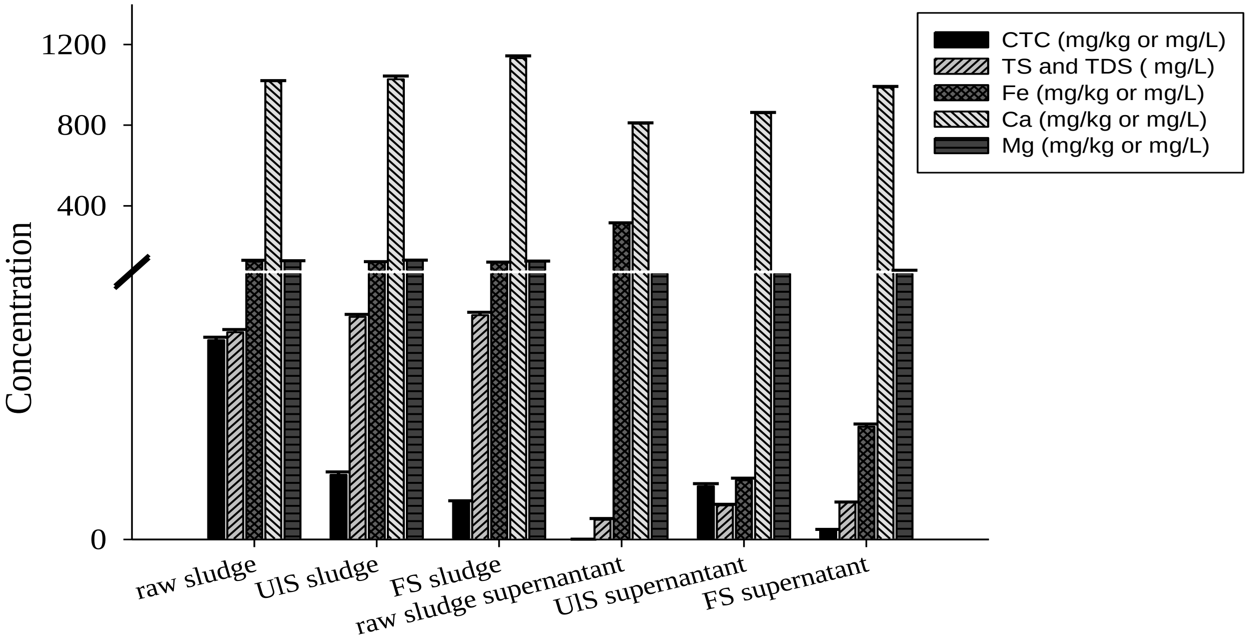
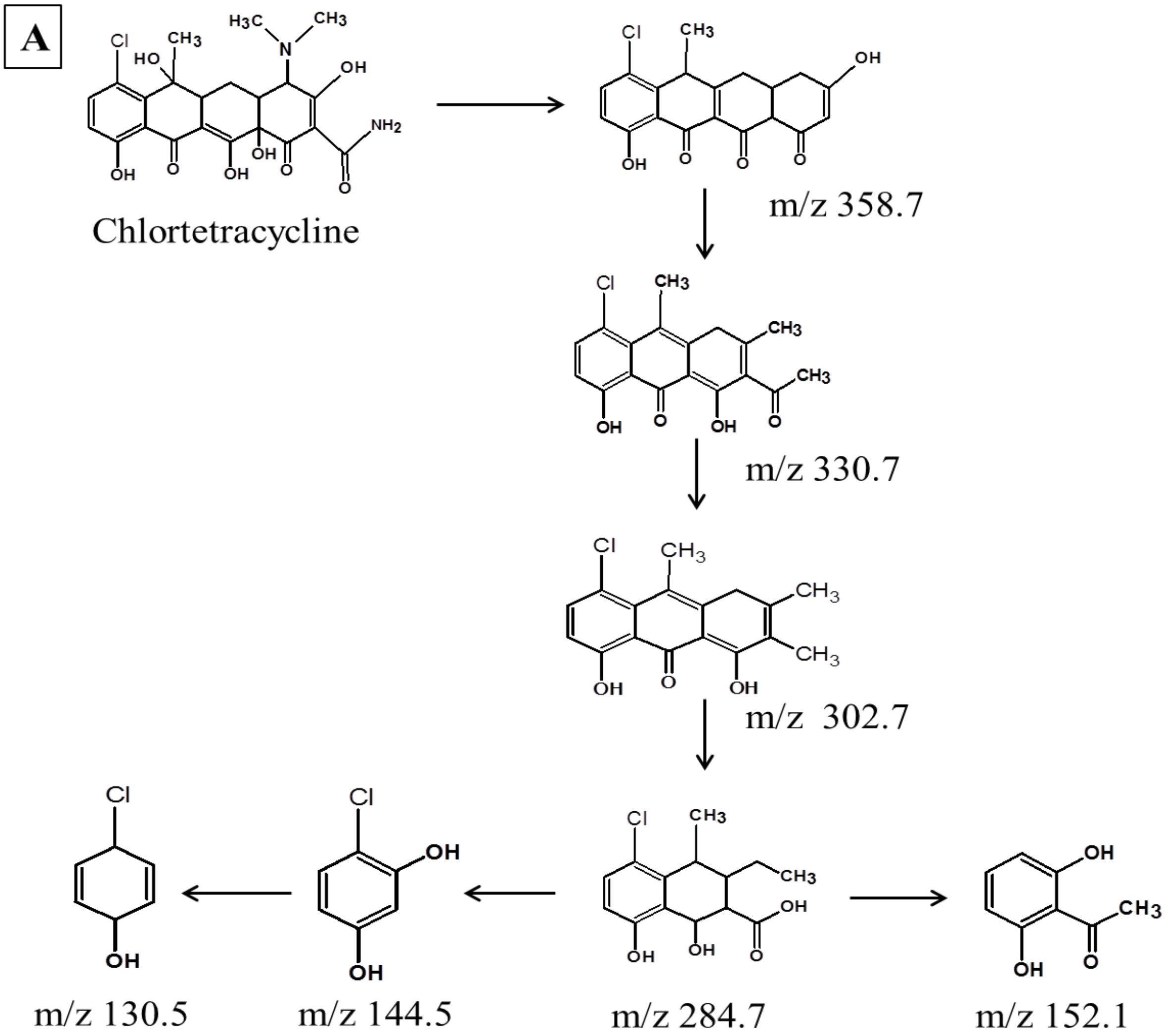


Figure 4.1.4 **Effect of metal and total solids on degradation of CTC in solid sludge and supernatant of treated wastewater sludge.**


A
Chlortetracycline
m/z 358.7
m/z 330.7
m/z 302.7
m/z 130.5
m/z 144.5
m/z 284.7
m/z 152.1

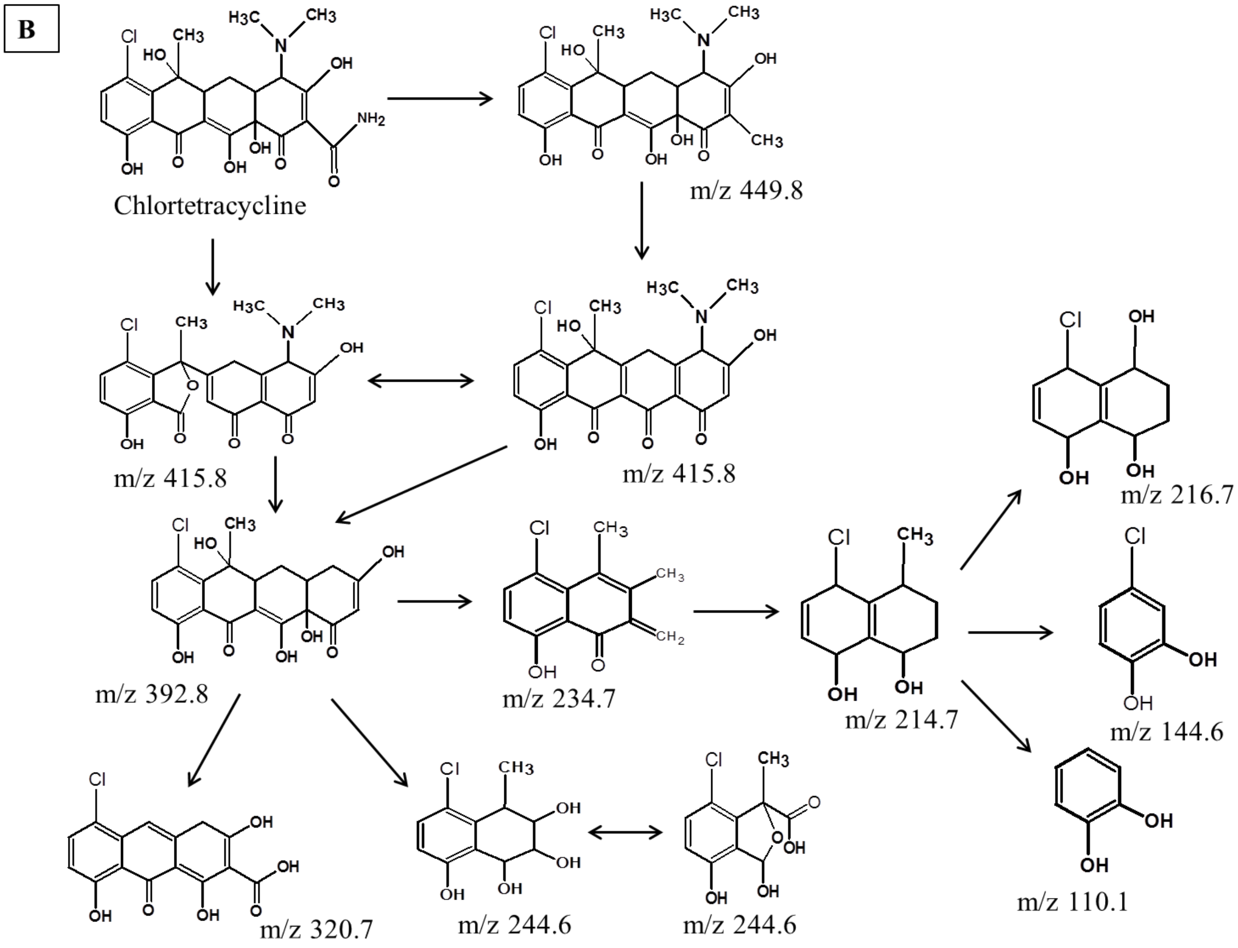


Figure 4.1.5 **Chemical structures and proposed degradation pathway of chlortetracycline and their degradation products: A) Fenton degradation; and B) Ferro-sonication degradation**

Appendix

$y = \beta_0 + \sum_{i=1} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j$ -- (1)

$d_{CTC\ sludge} = [\frac{CTC_{\ sludge} - B}{B - A}]$ -- (2)

$d_{CTC\ sludge} = 1\ if\ CTC_{\ sludge} \leq A$ --- (3)

$d_{CTC\ sludge} = 0\ if\ CTC_{\ sludge} > B$ --- (4)

$d_{CTC\ supernatant} = [\frac{CTC_{\ supernatant} - A}{A - B}]$ -- (5)

$d_{CTC\ supernatant} = 0\ if\ CTC_{\ supernatant} \leq A$--- (6)

$d_{CTC\ supernatant} = 1\ if\ CTC_{\ supernatant} > B$--- (7)

$d_{TDS} = [\frac{CTC_{\ TDS} - A}{A - B}]$ -- (8)

$d_{TDS} = 0\ if\ CTC_{\ TDS} \leq A$-- (9)

$d_{TDS} = 1\ if\ CTC_{\ TDS} > B$-- (10)

$d_{Energy} = [\frac{CTC_{\ Energy} - B}{B - A}]$ --- (11)

$d_{Energy} = 1\ if\ CTC_{\ Energy} \leq A$--- (12)

$d_{Energy} = 0\ if\ CTC_{\ Energy} > B$--- (13)

$D = (d_{CTC\ sludge} \times d_{CTC\ supernatant} \times d_{TDS} \times d_{Energy})^{1/4}$------------------------------------- (14)

$Ultrasonic\ intensity = \frac{\frac{Joules}{time}}{UlS\ probe\ diameter}$ --------------------------------------- (15)

$\eta = \frac{P_{Cal}}{P_{Elec}}$ ---------------------------------- (16)

$P_{Cal} = P_{UlS} = \left(\frac{dT}{dt}\right) t_{US}.C_p.M_{sludge}$----------------- (17)

$E \propto A^2$---------------------------- (18)

PART 2

DEGRADATION KINETICS OF CHLORTETRACYCLINE IN WASTEWATER USING ULTRASONIC ASSISTED LACCASE

Rama Pulicharla[a], Ratul Kumar Das[a], Satinder Kaur Brar[a*], Patrick Drogui[a], Rao Y. Surampalli[e]

[a] INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9.

[b] Department of Civil Engineering, University of Nebraska-Lincoln, N104 SEC PO Box 886105, Lincoln, NE 68588-6105, USA.

* Corresponding author: INRS-ETE, Téléphone: + 418 654 3116; Fax: + 418 654 2600, E -mail: satinder.brar@ete.inrs.ca

Résumé

L'antibiotique chlorétatracycline (CTC) est largement utilisé en médecine vétérinaire et est considéré comme un polluant récalcitrant. Cependant, sa quantité résiduelle dans l'environnement est un problème car elle pourrait contribuer au développement de bactéries résistantes. Dans cette étude, la CTC a été traitée avec de la laccase du champignon de la pourriture blanche, *Trametes versicolor* combiné avec des ultrasons. La chromatographie liquide-spectrométrie de masse a démontré que la CTC a été éliminée à plus de 80% en 2h lorsque le traitement par la casse à ultrasons (UAL) a été utilisé alors que la laccase seule prenait 2 jours pour dégrader 87% de la CTC. Le pH 4,5 a été considéré comme un pH optimal pour le traitement UAL. Le taux de dégradation a été augmenté de 14 fois lorsque l'ultrason a été combiné avec une dégradation de la lactate. Par rapport au traitement utilisant seule la laccase, la technique UAL a non seulement augmenté la dégradation de la CTC (~ 85%), mais aussi a réduit le temps de dégradation (2h). Les résultats obtenus mettent en évidence une efficacité améliorée de la dégradation de la CTC et la stabilité de la laccase dans l'UAL qui peut être utilisé pour oxyder un autre groupe d'antibiotiques de la tétracycline. En outre, les traitements de la laccase et de l'UAL présentaient des produits de dégradation similaires et aucune activité oestrogénique.

Mots clés: Chlorétatracycline; Dégradation; Laccase; Toxicité; Ultrasonication.

Abstract

Chlortetracycline antibiotic is widely used in veterinary medicine and is considered as a recalcitrant pollutant. However, its residual amount in the environment is an issue since it could contribute to the development of bacterial resistance. In this study, chlortetracycline (CTC) of 2 mg/L was treated with laccase from the white rot fungi, Trametes versicolor combined with ultrasonication. Liquid chromatography-mass spectrometry demonstrated that over 80% of CTC was removed in 2h when laccase treatment was assisted with ultrasonication while laccase treatment alone took 2 days to degrade 87% of CTC. And degradation rate was increased by 14 folds when ultrasonication was combined with laccase. Further, pH effect on ultrasonic assisted laccase (UAL) treatment efficiency and laccase activity was studied and pH 4.5 was found to be optimum for UAL treatment. Compared to the laccase treatment alone, the UAL technique not only increased CTC degradation (~80%) but also reduced degradation time (2h). The obtained results highlighted the enhanced degradation efficiency and stability of laccase in UAL which can be used for oxidizing another tetracycline group of antibiotics. Moreover, laccase and UAL treatments showed similar degradation products and no estrogenic activity.

Keywords: Chlortetracycline; Laccase; Ultrasonication; Degradation; Toxicity

Introduction

Rapid increase in population, income, and urbanization has driven the global consumption of milk and meat in developing countries (Delgado, 2003). Current industrial farming with extensive use of antibiotics is likely helping to meet the growing demand of animal products. As a result, animal farming became a key source for continuous release of antibiotics into the environment.

Even though the use of antibiotics as feed additive in animals was started as early as it did for humans, their current intake in animals has exceeded the human consumption(Halloran, 2012). This is due to their massive usage for growth promotion and disease prevention in animals apart from treating infections. Apart from this, up to 70-90% of antibiotics are passed through animal excretions into the environment. This heavy usage and excretion of antibiotics accelerate antibiotic resistance transmittance from animal food to humans. Specifically, the wide usage of broad spectrum antibiotics for all bacterial infections contributes to the development and spread of non-targeted microorganism's resistant strains(Smith *et al.*, 2002) which result in multiple resistance.

Conventional biological treatment plants are not designed for effective removal of low concentration (ug/L-ng/L) contaminants including antibiotics. Antibiotics at these low concentrations adversely affecting the biological treatment processes and other pollutant biotransformation processes(Campos *et al.*, 2001, Gomez *et al.*, 1996). And further, the advanced physical and chemical methods, such as ultraviolet treatment, ozone treatment, adsorption and membrane filtration processes have been proven to some extent inefficient in the removal of antibiotics(Oller *et al.*, 2011). However, research is continuously growing on these technologies to make them low cost, more efficient, wider applications, safe and easy operation. There is an evidence on the frequent occurrence of a wide variety of antibiotics and their metabolites in a wide range of water sources including surface water, drinking water, and ground water across the world (Watkinson *et al.*, 2009). Currently applying technologies, such as ultraviolet and ozone treatments to wastewater (WW) might be efficient for some contaminants; still, the toxicity, residual concentration, and mineralization of contaminants are the deciding factors of treatment efficiency(Jiao *et al.*, 2008, Li *et al.*, 2008).

In fact, currently, researchers are putting efforts to develop treatment systems using enzymes to remove pollutants before releasing the wastewater effluent into the environment. It is well known that the extracellular ligninolytic enzymes (laccase, lignin peroxidase, and manganese peroxidase) of white rot fungi have great potential in degrading environmental pollutants(Mester *et al.*, 2000). Laccase belongs to the oxidases family of enzymes which oxidizes many organic contaminants having phenolic and non-phenolic aromatic structures(Rodrıguez *et al.*, 2004). Former research points out that non-selectivity of laccase towards contaminants is efficient in removing different classes of antibiotics including, tetracyclines, sulfonamides, quinolones, penicillins and fluoroquinolones (Ding *et al.*, 2016). Laccase oxidation without any mediator showed lower degradation efficiency and slow degradation rate(Johannes *et al.*, 1996). These drawbacks can be overcome by simultaneous application of ultrasonication with enzymes to increase the degradation rate (Sutar *et al.*, 2015).

In ultrasonic phenomenon, generation and growth of cavities and their subsequent collapse generate heat and hydroxyl radicals(Gogate *et al.*, 2009). This causes turbulence in the media which enhances mass transfer rates and liquid circulation. Similarly, thermal and mechanical energies associated with ultrasonication lead to oxygen mass transfer which is essential to speed up enzymatic reduction reactions to degrade contaminants. Apart from this, a portion of ultrasonic energy absorbed by the medium is converted into heat and increase media temperature which increases the enzymatic activity. Studies have reported the enhancement of catalytic activity, the stability of enzymes and longevity of catalytic activity with the use of ultrasonic process(Shah *et al.*, 2008).

Herein, crude laccase produced by *Trametes versicolor* was used to degrade chlortetracycline (CTC) which was selected as typical veterinary antibiotic having wider application and less biodegradable nature. An agro-industrial waste apple pomace was used as a substrate to produce laccase. So far, the application of ultrasound assisted laccase (UAL) catalyzed degradation of CTC has not been reported previously. In this context, the ultrasonic enhanced catalytic activity of laccase to degrade CTC was evaluated in wastewater. In addition, different operating parameters, such as pH and enzyme stability during ultrasonication were studied to determine optimum operating conditions for CTC degradation. Further, transformation products were identified by liquid chromatography-mass spectrometry (LC-MS/MS) and estrogenic activity of resulting treated streams was also determined in WW.

Materials and methods

Chemicals

Chlortetracycline hydrochloride was purchased from Toronto Research Chemicals (Toronto, Canada). Methanol (HPLC grade), tween 80, disodium hydrogen phosphate (Na_2HPO_4), citric acid, sodium azide were purchased from Fisher Scientific (Ontario, Canada). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Sigma-Aldrich. Millipore system (Milford, MA, USA) Milli-Q/Milli-RO was used for HPLC grade water preparation.

Wastewater sample

Wastewater samples were collected from wastewater treatment plant (WWTP) of Quebec Urban Community (CUQ) (Sainte-Foy, Quebec City, QC, Canada), with a sewage treatment capacity of 13,140 m^3/h. The plant treats the sewage with primary and physical–chemical treatments and further discharges wastewater into the Saint-Lawrence River. WW samples used in this study were collected before UV treatment and stored under dark conditions at 4 ± 1 ^oC to prevent CTC degradation. The basic characteristics of WW used in this study are presented in table 4.2.1.

Solid state fermentation

Microorganism and fermentation: White-rot fungus *T. versicolor* (ATCC 20869) was selected for solid state fermentation (SSF) to produce laccase. Potato dextrose agar petri plates were used to inoculate the fungus and incubated for 7 days at 30 ± 1 °C. The culture plates were stored at 4 ± 1 °C and sub-cultured for every 4 weeks.

Crude laccase production: Erlenmeyer flask (500 mL) containing 20 g of dried apple pomace containing Tween 80 (0.1%) and moisture content 75% (w/w) was sterilized at 121 °C for 15 min under 1 atm pressure. Subsequently, the culture medium was inoculated with the mycelia of *T. versicolor* followed by incubation for 14 days at 30 ± 1 °C (Ajila *et al.*, 2011). Apple pomace was stored at -20 °C, until use for its conservation.

After incubation, 200 mL of pH 6.5 sodium phosphate buffer laccase was added to the flask and mixed manually, then left to stirring at 250 rpm for 1 h to extract enzyme into the buffer. The supernatant was collected by centrifugation at 6500 × *g* for 20 min at 4 °C and stored at 4 ± 1 °C.

Enzyme assay: Laccase activity was measured based on the oxidation of ABTS substrate (500 mM) at 420 nm ($E_{max} = 3.6 \times 10^4 M^{-1}\ cm^{-1}$). The reaction media having 0.1 mL laccase extract in 2.4 mL sodium phosphate-citrate buffer (pH 3.0) and 0.5 mL of ABTS (1.5 mM) were incubated at 45 °C for 10 min to measure the activity(More *et al.*, 2011). One unit laccase activity is equivalent to 1.0 μmol of product formation per min under the assay condition.

Enzyme stability: Laccase activity was tested during degradation studies at room temperature 21±2 ^{o}C for 48 h. And also, kinetics of laccase activity during ultrasonication and temperature alone was tested with the respective pH degradation study of CTC. The specific amount of laccase was transferred immediately into pH 3.0 sodium phosphate-citrate buffer at each time and stored at 4 ± 1 °C, until laccase activity measurement.

Experimental procedure: About 2 mg/L CTC spiked in WW was tested for UAL degradation. Laccase degradation kinetics of CTC was carried out for 72 h at room temperature, 150 rpm, pH 6±0.5 using 0.5 IU laccase activity. Bath ultrasonication was carried for 2h without temperature control at pH 6±0.5 to find CTC degraded by ultrasonication. Combined ultrasonication and laccase degradation were performed at different pH including 3.0, 4.5, 6.0, 8.0 and 10.0 at constant 0.5 IU laccase activity and CTC concentration (2 mg/L) spiked in WW.

Analysis of CTC and its transformation products

Following laccase and ultrasonic assisted laccase degradation of CTC in wastewater, laccase activity was quenched by adding 0.1mM NaN_3 and further samples were diluted with methanol and stored at 4 ^{o}C in dark.

To analyze the CTC concentration in treated samples, LC-MS/MS (Thermo fisher scientific) coupled to a 4000 TSQ Quantum Access Mass Spectrometer equipped with an electrospray ionization source was used. Chromatographic separations were carried out in a reverse-phase Hypersil Gold C18 column (100 x 2.1mm, 3µm particle size). The mobile phase used for elution of CTC consists of 0.1% acetic acid in MilliQ water (A) and 0.1% acetic acid in acetonitrile (B). Gradient elution technique used was as follows: 0 min, 90% A (10% B); 0–12 min, 30% A (70% B); and 12–18 min, 90% A (10% B), at a flow rate of 230 µL/min, 20 µL injection volume and column temperature of 30 °C. Electrospray ionization in positive ion mode was operated in the mass spectrometer and CTC acquisition was monitored by using multiple reaction transitions. Other parameters used were as follows: spray voltage, 4000 V; collision gas-Argon, 1.7 mTorr; capillary temperature, 340°C. The internal standard method was used to quantify the CTC. The five-point calibration curve (2 µg/L to 100 µg/L) was prepared from a stock solution of 500 µg/L CTC and sulfamethazine was used as internal standard.

The experiments were run in LC–MS/MS both positive and negative electrospray ionization modes with a mass scan range of m/z 50−1000 to identify the Transformation products (TPs) of CTC with the same column. These experiments were carried out in samples with (i) real WW without spiking CTC; (ii) WW with laccase (without spiking CTC); (iii) laccase degraded CTC; and UAL degraded CTC spiked in WW. A comparison was done between laccase and UAL TPs in order to identify the difference in the formed TPs.

Yeast Estrogen Screen (YES) assay

The estrogenic activity of untreated and treated CTC in WW samples (enzymatic and ultrasonic assisted enzymatic) were tested using YES assay described by Routledge and Sumpter (1996) (Routledge *et al.*, 1996). In this YES assay, yeast strain is genetically modified to express the human estrogen receptor (hER) in an estrogen-dependent manner. Experiments were carried out in a 96-well plate (Costar Brand, NY, USA). All standards (17β-estradiol), stock solutions of CTC and samples were diluted with 70% alcohol prior to YES assay to prevent unwanted bacterial growth. Aliquots of 10 µL of each standard, stock, and samples diluted in alcohol were transferred to a 96-well plate under laminar flow chamber. The alcohol aliquots placed in 96 well was allowed to completely evaporate and further 200 µL aliquots of the yeast assay medium containing the recombinant yeast cells (hER-transfected) and chlorophenol red-β-d-galactopyranoside (CPRG) were placed after evaporation. Each plate contains one row of blanks (assay medium and alcohol evaporated) and a standard curve for standard 17β-estradiol. The plates were sealed with parafilm and shaken at 200 rpm for 5 min to mix assay contents yeast cells and further 3 days static incubation at 32±1 ^{o}C. Periodically the color development of the essay medium in plates was checked using a multi-reader microplate spectrophotometer (Epoch, BioTek, USA) at an absorbance of 540 nm.

Statistical analysis

All experiments were performed in triplicate. Statistical significance tests were performed using ANOVA (analysis of variance). *P*-values less than 0.05 were considered statistically significant. Zero, first and second order kinetics were calculated to find out the degradation rate (Table 4.2.2).

Results and discussion

Individual laccase and ultrasonic CTC degradation

Considering the cost-effective aspect of using enzymes at industrial level in degrading pollutants, current research has accelerated on production methods of enzymes and augmentation of their activity by combining with other technologies (Kurniawati *et al.*, 2007, Pandey *et al.*, 1999). To reduce the cost of enzymes production, apple pomace industrial waste was used as a substrate for solid fermentation to produce crude laccase enzyme for further degradation studies. Optimized incubation conditions from previous studies, such as 37 ± 1 ^{o}C for 14 days were used for laccase production(Ajila *et al.*, 2011). Significantly (p>0.05) negligible production of lignin peroxidase and manganese peroxidase was detected in the crude enzyme extract.

Initial laccase degradation studies of CTC were carried out in 2 mg CTC/L spiked in WW using laccase of 0.5 U/L activity at room temperature, pH 6 and agitation speed of 150 rpm for 48h. Degradation kinetics of CTC in WW is depicted in figure 4.2.1. About 10% degradation was observed for CTC at room temperature in 48h (presented as supplementary data). It has been observed that 87±2% of CTC was degraded in 48h without any mediators. Similar results were reported in previous studies of Suda et al., 2012 (Suda *et al.*, 2012) where 48% CTC degradation with laccase alone in 4h and 50% in 5h was recorded in this study. Table 4.2.2 provides the rate constants and correlation coefficients of different kinetic models for laccase degradation of CTC.

The second-order kinetic model described very well the degradation process of CTC by laccase oxidation. This kinetic behavior might be due to the concentration of laccase and CTC seems to be affecting the degradation rate of CTC. Studies of Yang et al., 2017 (Yang *et al.*, 2017) showed that increased enzyme loading increased the degradation and also different degradation efficiency among tested compounds due to varied transformation efficiency with laccase. Laccase was found to be stable ($p < 0.05$) after two days at room temperature. Sonication was performed to degrade CTC spiked in WW for 2h under similar conditions to enzymatic degradation. Sonication studies showed 18±5% of CTC degradation. Most of the literature on bath ultrasonication of CTC was focused on its extraction from complex media for 15-30 min (Zhou *et al.*, 2009). In this study, 2h sonication showed 18±5% of CTC degradation which is not economically feasible to use ultrasonication (UlS) alone to degrade CTC in WW. Studies used mediators, such as 1-hydroxybenzotriazole and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and immobilization techniques were used to speed up laccase oxidation. Increase in catalytic activity of laccase without the help of chemical mediator is preferable and advantageous because the effect of mediators on laccase oxidation varies with formed radicals; stability of laccase and furthermore mediators may be toxic and costly and might inactivate laccases (Ashe *et al.*, 2016, Kurniawati *et al.*, 2007, Morozova *et al.*, 2007). This study pursues further ultrasonication process as a mediator to speed up the degradation process of CTC by laccase oxidation.

Ultrasonic assisted laccase degradation

To investigate the combined effect of ultrasonication and enzyme on CTC degradation, spiked WW with CTC was treated with 0.5 U/L laccase and bath sonication for 2h. As shown in figure 4.2.2, UAL showed degradation of over 60.2% of CTC under similar conditions to individual laccase (31±3%; 2h; figure 4.2.1) and (UlS) degradation

(18±5%; 2h; figure 4.2.2). UAL degradation showed two and three folds increase in degradation efficiency compared to laccase and UlS degradation, respectively. Changes in temperature of wastewater during sonication are presented in supplementary data Increased temperature (25 to 52 ^{o}C) during UlS and also the enhanced oxygen transfer rates might be responsible for the higher degradation efficiency in case of UAL. During laccase catalysis, molecular oxygen acts as a terminal electron acceptor in copper one-electron redox reactions to form two molecules of water.

Effect of pH on CTC degradation and laccase activity during the UAL

pH is an important parameter affecting the degradation of CTC in view of enzyme workability as well as ionization state of CTC. Enzymes need specific pH to retain its active conformation for its activity and selectivity. Therefore, considering these facts, to find out the optimum pH of UAL degradation, five different pHs including 3, 4.5, 6, 8 and 10 have been studied. Effect of pH on degradation kinetics of CTC at different pH is shown in Figure 4.2.2. pH 4.5 showed highest degradation of CTC around 79%, further 60% and 20% CTC was degraded at pH 6.0 and 3.0, respectively. No significant degradation of CTC was observed at alkaline pH of 8 and 10. Laccase activity was simultaneously measured during ultrasonication at different studied pH and results are presented in figure 4.2.3. Bath ultrasonication increased the laccase activity by 20% at pH 6.0 whereby the enzyme activity remained the same at pH 4.5 after 2h. At pH 3 and 8, laccase activity was reduced by 50% and 70%, respectively and the enzyme was deactivated at pH 10.

Results showed that extent of degradation was lower at acidic (pH 3) and alkaline pH (pH>8) while at pH 4.5, higher degradation was obtained. If the degradation efficiency was compared with laccase activity, at acidic pH 3, enzyme activity was reduced to 50% and the corresponding CTC degradation was 20%. At pH 3, UlS process was able to produce more OH radicals (Pulicharla *et al.*, 2017) which could possibly target the enzyme which are bigger molecules when compared to smaller CTC. And the laccase activity at pH 3 after 2h was reduced to half which explains that the enzyme became inactivated with time at pH 3. At higher pH >8, laccase is inactivated (figure 3) which results in no significant degradation of CTC. Laccase activity after UAL degradation was compared with the results of temperature alone effect on laccase activity presented in supplementary data. The increase in temperature enhances the laccase activity significantly ($p<0.05$) in the case of pH 4.5 and 6 wherein UlS it was observed only at pH 6. Hence, at lower pH, UlS was affected the laccase activity. The optimum pH was found to be 4.5 where highest CTC degradation (79%) was observed. At this pH, CTC exists in ionized form and laccase activity was found to remain the same throughout the experimental time (2h). In contrast, at pH 6, CTC exists as zwitterionic form and laccase activity was increased by 20%, but degradation was found to be lower than pH 4.5. This indicated that at lower pH 4.5, the ionized form of CTC was more susceptible to degrade compare to zwitterionic form. No increase in laccase activity at pH 4.5 was due to the production of OH radicals which might be degrading the enzyme, but the effect was lower than pH 3.0 (Pulicharla *et al.*, 2017).

Considering the degradation kinetics of laccase and UAL treatments, all followed the second order kinetics except pH 4.5 UAL treatment (Table 4.2.2). This can be explained by no change in the enzyme activity at pH 4.5. On comparing the degradation rates of all studied experiments with second order kinetics, pH 4.5 showed a higher rate of degradation 1.83 $M^{-1}h^{-1}$ and 3 and 10 folds reduced rate at pH 6 and 3, respectively. When laccase and UAL treatment were compared, UAL treatment showed 14 folds higher degradation rate. UAL treatments for other contaminants showed higher degradation rate similar to current study(Tauber *et al.*, 2005). This study concluded that in UAL degradation, UlS enhanced the laccase oxidation by increasing the media temperature this might be mainly due to the increased oxygen mass transfer which is essential to speed up laccase activity and also further interaction between laccase and CTC molecules is enhanced by UlS cavitation.

Identification of CTC transformation products and proposed degradation pathways

The possible part of CTC that was vulnerable to reduction reactions and the summary of proposed degradation pathways are presented in figure 4.2.4. This study is reporting for the first time the transformation products of CTC by UAL degradation. Laccase and UAL degradation showed similar oxidation sites, such as C2, C3, C4, and C6 (Figure 4.2.4A) for CTC. Laccase treatment showed 6 TPs having *m/z* 480.9, 430.8, 413.8, 41.9, 352.8 and 260.7 where UAL degradation showed 8 TPs of *m/z* 444.8, 437.8, 413.8, 403.9, 363.8, 260.7, 240.6, and 228.6. Similar TPs products, such as 413.8 and 260.7 were observed in both treatment processes indicating the similar degradation products. Additionally, the formation of smaller TPs, such as *m/z* 240.6, and 228.6 in UAL treatment indicated the potential of UAL degradation to form persistent end products. TPs are formed by hydrogenation (480.9), dehydroxylation (444.8) and demethylation (430.7), decarboxylation and de-ammoniation (451.9) reactions. These generated TPs involve redox reactions at C2, C3, C4, and C6 sites and this has been reported in previous studies of tetracycline degradation by laccase (Llorca *et al.*, 2015, Yang *et al.*, 2017). To the best of authors' knowledge, these TPs have been detected for the first time during the UAL of CTC.

Estrogenic activity of laccase degraded CTC in WW samples

Figure 4.2.5 shows the response of the estrogenicity to the enzyme, ultrasonication, ultrasonic assisted laccase-treated WW spiked and unspiked CTC. The blank (alcohol) samples absorbance range remained within 1 to 1.2 range after a 3-day incubation. Figure 4.2.5 showed that WW spiked CTC before and after treatment with different methods has no estrogenic activity compared to 17β-estradiol. The estrogenic samples are red coloured with 17β-estradiol and other samples including blank (alcohol), WW spiked and unspiked CTC samples did not show any color change from yellow to red. Even though the TPs products were not completely similar, resulting laccase and UAL treatment samples were not estrogenic. These results are similar to decreased estrogenic effect of other chlorinated endocrine disrupting compounds which are degraded by laccase (Cajthaml, 2015). This might be due to the similar degradation mechanisms, such as C-C and C-O bond oxidation which might be carried out during degradation of contaminants with electron rich aromatic structures and chlorinated phenols(ten Have *et al.*, 2001). Hence, CTC having aromatic rings, electron rich functional groups and chlorinated phenols can be efficiently degraded by laccase and showed no estrogenicity of resulting TPs.

Conclusion

The combination of laccase with ultrasonication was studied to degrade CTC in wastewater. pH of UAL affecting the degradation was optimized along with identification of the TPS during the degradation process. Laccase at 0.5 U/L showed 87% CTC elimination in 2 days at pH 6, 25 ^{o}C whereas UAL degraded over 60% of 2 mg/L CTC in 2h. Optimum pH for UAL treatment was found to be 4.5 where 80% of CTC was degraded. Further, UAL showed 14 folds increase in degradation rate compared to laccase alone degradation. Even though the degradation efficiency and rate were different, both treatments have shown similar degradation products and no estrogenic activity. Enhanced degradation and no toxic TPs state its importance in degrading other contaminants. This study signified the fact that UAL treatment is a promising technique which can improve degradation within shorter time.

Acknowledgement

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Discovery Grant 355254 and Strategic Grant), and Ministère des Relations Internationales du Québec (coopération Québec-Catalanya 2012–2014, 01.801-23525) for financial support. Authors are also thankful to “merit scholarship program for foreign students” (FQRNT) for financial assistance to Rama Pulicharla. Thanks to Prof. Viviane Yargeau, from McGill University for providing YES test yeast strain to perform estrogenicity study.

References

Ajila C, Brar S, Verma M, Tyagi R & Valéro J (2011) Solid-state fermentation of apple pomace using Phanerocheate chrysosporium–Liberation and extraction of phenolic antioxidants. *Food Chemistry* 126(3):1071-1080.

Ashe B, Nguyen LN, Hai FI, Lee D-J, van de Merwe JP, Leusch FD, Price WE & Nghiem LD (2016) Impacts of redox-mediator type on trace organic contaminants degradation by laccase: Degradation efficiency, laccase stability and effluent toxicity. *International Biodeterioration & Biodegradation*.

Cajthaml T (2015) Biodegradation of endocrine-disrupting compounds by ligninolytic fungi: mechanisms involved in the degradation. *Environmental microbiology* 17(12):4822-4834.

Campos JL, Garrido JM, Méndez R & Lema JM (2001) Effect of two broad-spectrum antibiotics on activity and stability of continuous nitrifying system. *Applied biochemistry and biotechnology* 95(1):1-10.

Delgado CL (2003) Rising consumption of meat and milk in developing countries has created a new food revolution. *The journal of nutrition* 133(11):3907S-3910S.

Ding H, Wu Y, Zou B, Lou Q, Zhang W, Zhong J, Lu L & Dai G (2016) Simultaneous removal and degradation characteristics of sulfonamide, tetracycline, and quinolone antibiotics by laccase-mediated oxidation coupled with soil adsorption. *Journal of hazardous materials* 307:350-358.

Gogate PR & Kabadi AM (2009) A review of applications of cavitation in biochemical engineering/biotechnology. *Biochemical Engineering Journal* 44(1):60-72.

Gomez J, Mendez R & Lema JM (1996) The effect of antibiotics on nitrification processes. *Applied biochemistry and biotechnology* 57(1):869-876.

Halloran J (2012) The overuse of antibiotics in food animals threatens public health. *Consumer union. Policy & action for consumer reports.–2012.–режим доступу: https://consumersunion. org/news/the-overuseof-antibiotics-in-food-animals-threatens-public-health-2*.

Jiao S, Zheng S, Yin D, Wang L & Chen L (2008) Aqueous photolysis of tetracycline and toxicity of photolytic products to luminescent bacteria. *Chemosphere* 73(3):377-382.

Johannes C, Majcherczyk A & Hüttermann A (1996) Degradation of anthracene by laccase of Trametes versicolor in the presence of different mediator compounds. *Applied Microbiology and Biotechnology* 46(3):313-317.

Kurniawati S & Nicell JA (2007) Efficacy of mediators for enhancing the laccase-catalyzed oxidation of aqueous phenol. *Enzyme and microbial technology* 41(3):353-361.

Li K, Yediler A, Yang M, Schulte-Hostede S & Wong MH (2008) Ozonation of oxytetracycline and toxicological assessment of its oxidation by-products. *Chemosphere* 72(3):473-478.

Llorca M, Rodríguez-Mozaz S, Couillerot O, Panigoni K, de Gunzburg J, Bayer S, Czaja R & Barceló D (2015) Identification of new transformation products during enzymatic treatment of tetracycline and erythromycin antibiotics at laboratory scale by an on-line turbulent flow liquid-chromatography coupled to a high resolution mass spectrometer LTQ-Orbitrap. *Chemosphere* 119:90-98.

Mester T & Tien M (2000) Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants. *International Biodeterioration & Biodegradation* 46(1):51-59.

More SS, PS R & Malini S (2011) Isolation, purification, and characterization of fungal laccase from Pleurotus sp. *Enzyme research* 2011.

Morozova O, Shumakovich G, Shleev S & Yaropolov YI (2007) Laccase-mediator systems and their applications: a review. *Applied Biochemistry and Microbiology* 43(5):523-535.

Oller I, Malato S & Sánchez-Pérez J (2011) Combination of advanced oxidation processes and biological treatments for wastewater decontamination—a review. *Science of the total environment* 409(20):4141-4166.

Pandey A, Selvakumar P, Soccol CR & Nigam P (1999) Solid state fermentation for the production of industrial enzymes. *Current science* 77(1):149-162.

Pulicharla R, Brar SK, Rouissi T, Auger S, Drogui P, Verma M & Surampalli RY (2017) Degradation of chlortetracycline in wastewater sludge by ultrasonication, Fenton oxidation, and ferro-sonication. *Ultrasonics Sonochemistry* 34:332-342.

Rodrıguez E, Nuero O, Guillén F, Martınez A & Martınez M (2004) Degradation of phenolic and non-phenolic aromatic pollutants by four Pleurotus species: the role of laccase and versatile peroxidase. *Soil Biology and Biochemistry* 36(6):909-916.

Routledge EJ & Sumpter JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental toxicology and chemistry* 15(3):241-248.

Shah S & Gupta MN (2008) The effect of ultrasonic pre-treatment on the catalytic activity of lipases in aqueous and non-aqueous media. *Chemistry Central Journal* 2(1):1.

Smith DL, Harris AD, Johnson JA, Silbergeld EK & Morris JG (2002) Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proceedings of the National Academy of Sciences* 99(9):6434-6439.

Suda T, Hata T, Kawai S, Okamura H & Nishida T (2012) Treatment of tetracycline antibiotics by laccase in the presence of 1-hydroxybenzotriazole. *Bioresource technology* 103(1):498-501.

Sutar RS & Rathod VK (2015) Ultrasound assisted Laccase catalyzed degradation of Ciprofloxacin hydrochloride. *Journal of Industrial and Engineering Chemistry* 31:276-282.

Tauber MM, Guebitz GM & Rehorek A (2005) Degradation of azo dyes by laccase and ultrasound treatment. *Applied and environmental microbiology* 71(5):2600-2607.

ten Have R & Teunissen PJ (2001) Oxidative mechanisms involved in lignin degradation by white-rot fungi. *Chemical reviews* 101(11):3397-3414.

Watkinson A, Murby E, Kolpin D & Costanzo S (2009) The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. *Science of the total environment* 407(8):2711-2723.

Yang J, Lin Y, Yang X, Ng TB, Ye X & Lin J (2017) Degradation of tetracycline by immobilized laccase and the proposed transformation pathway. *Journal of Hazardous Materials* 322:525-531.

Zhou J, Xue X, Li Y, Zhang J, Chen F, Wu L, Chen L & Zhao J (2009) Multiresidue determination of tetracycline antibiotics in propolis by using HPLC-UV detection with ultrasonic-assisted extraction and two-step solid phase extraction. *Food Chemistry* 115(3):1074-1080.

Table 4.2.1 Characterization of wastewater used in this study

Parameter	**Value**
pH	7.5±0.3
Total solids (g/L)	0.57±0.01
Suspended solids (g/L)	0.32±0.08
Volatile solids (g/L)	0.17±0.12
Volatile suspended solids (g/L)	0.38±0.05
Total organic carbon (g/L)	0.005±0.8
Alkalinity (g/L)	0.15±0.09
Ammonical nitrogen (g/L)	0.04±0.38
Chlortetracycline (μg/L)	8.54±0.2

Table 4.2.2 Correlation coefficients and rate constants of different degradation techniques used to degrade chlortetracycline in wastewater

Treatment method		**Zero order**		**First order**		**Second order**	
		(R^2)	**K (M s^{-1})**	**(R^2)**	**K (s^{-1})**	**(R^2)**	**K (M^{-1} s^{-1})**
Enzymatic degradation		0.317	0.0456	0.829	0.0401	0.924	0.1297
Ultrasonic assisted enzymatic treatment with different pH	3	0.806	0.648	0.946	0.132	0.960	0.163
	4.5	0.698	1.157	0.977	0.765	0.922	1.83
	6	0.699	0.695	0.917	0.39	0.963	0.685
	8	0.788	0.433	0.781	0.037	0.778	0.0405
	10	0.340	0.343	0.314	0.317	0.317	0.0153

K-rate constant

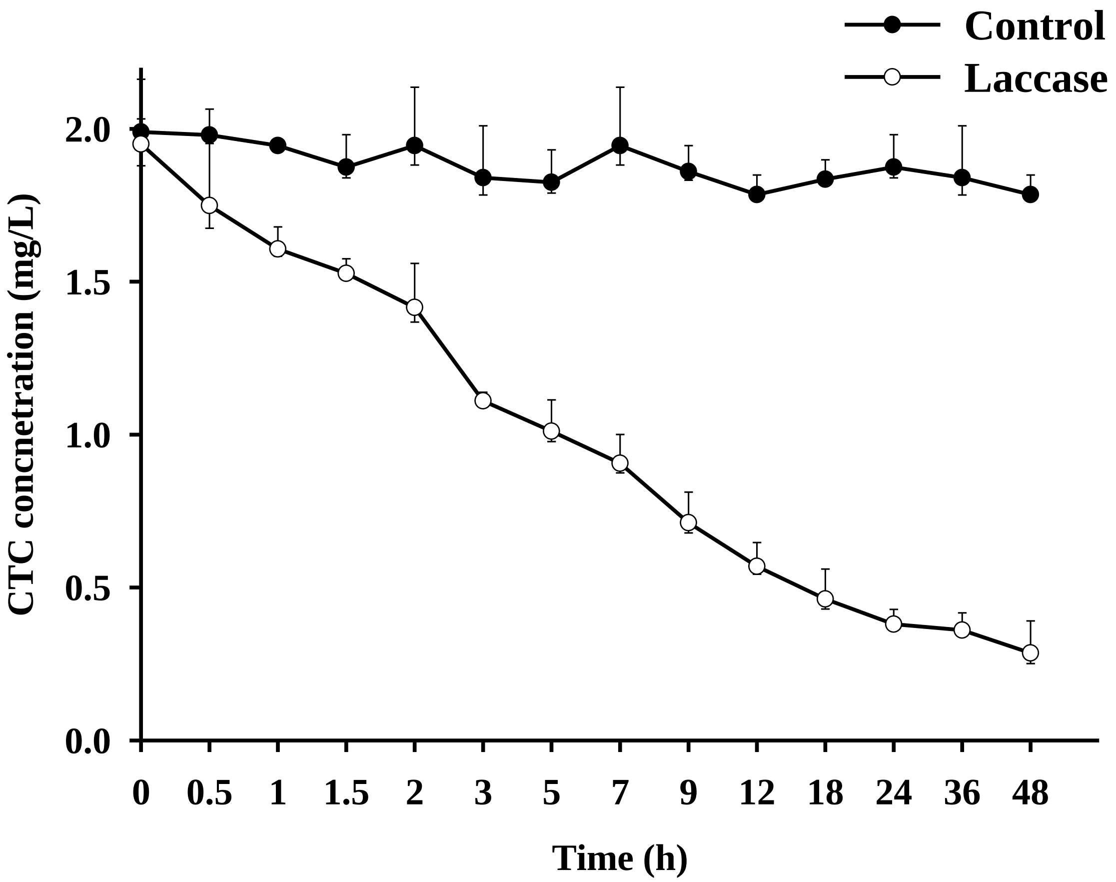


Figure 4.2.1 **Laccase degradation kinetics of chlortetracycline in wastewater at room temperature.**

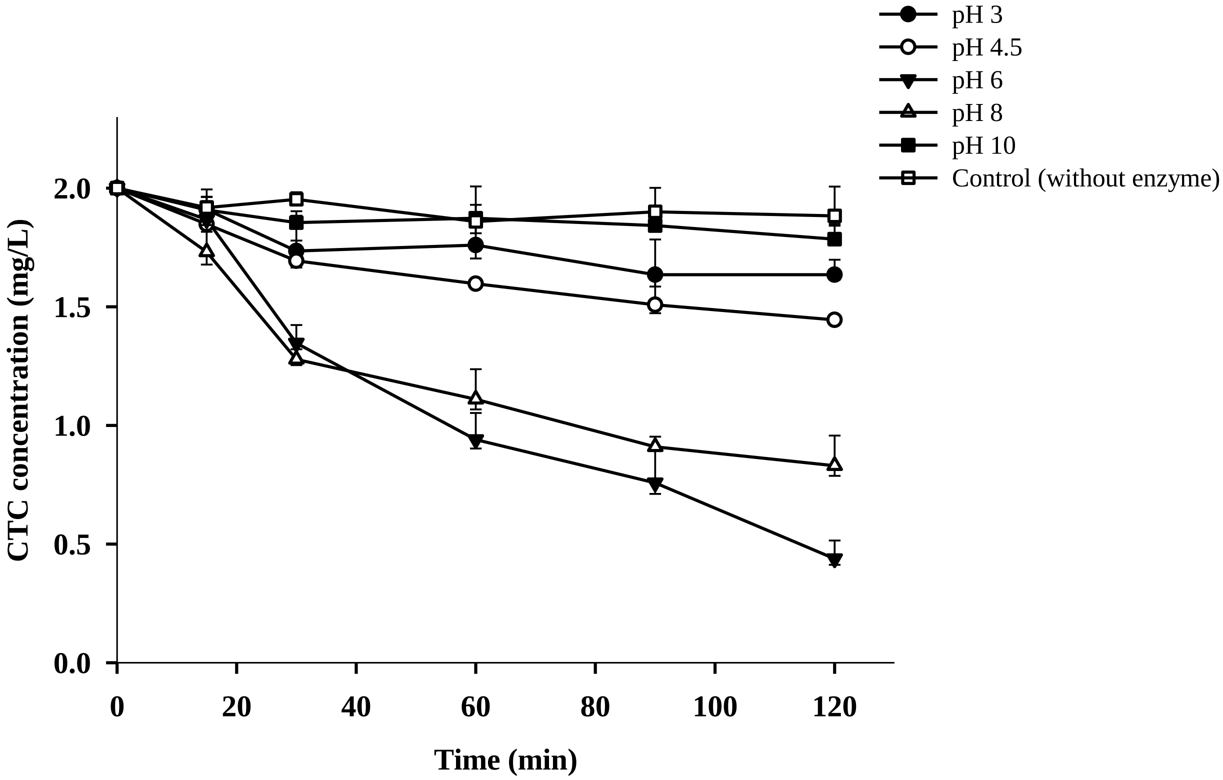


Figure 4.2.2 **Effect of pH on ultrasonic assisted degradation kinetics of chlortetracycline in wastewater at 0.5 IU laccase activity and control without enzyme at pH 6.0±0.5**

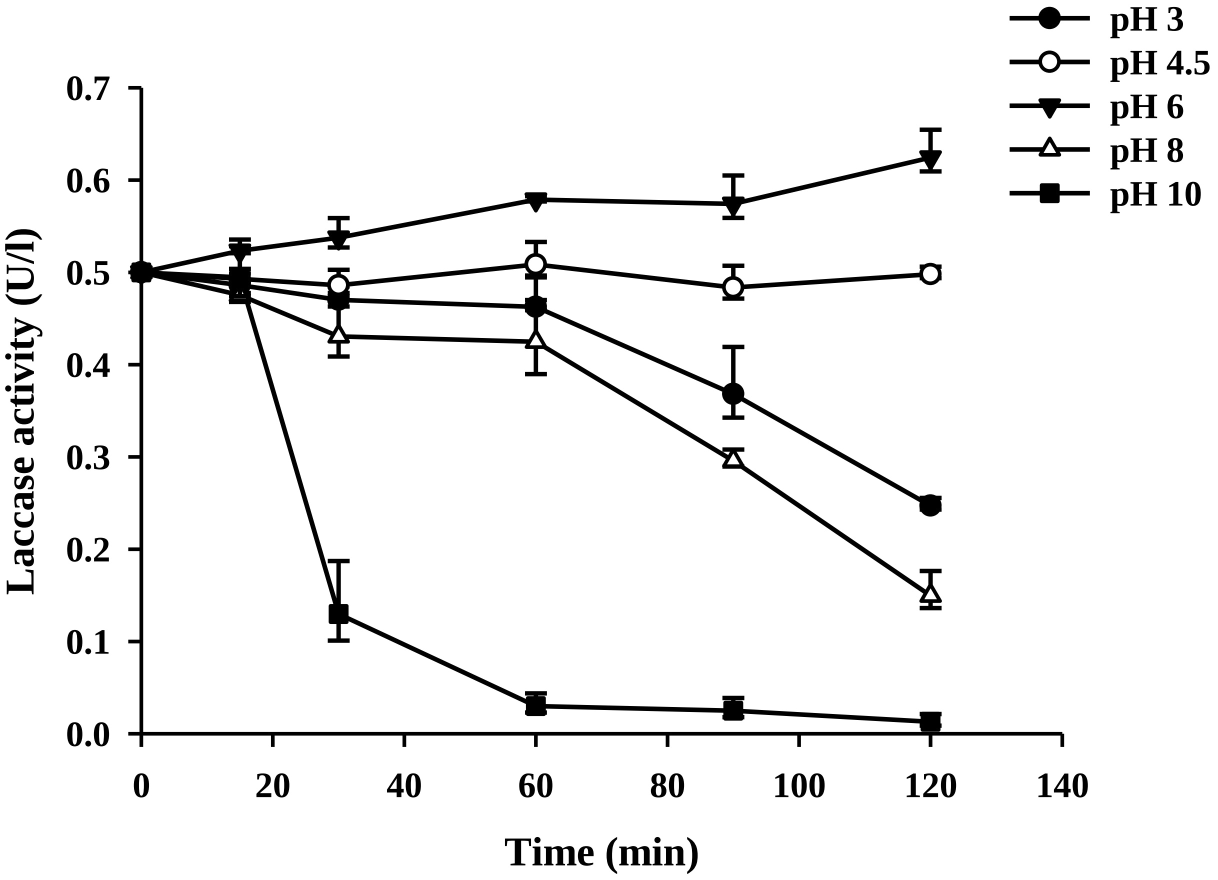


Figure 4.2.3 **Effect of pH on stability kinetics of laccase (0.5 IU) during ultrasonic assisted degradation of chlortetracycline in wastewater**

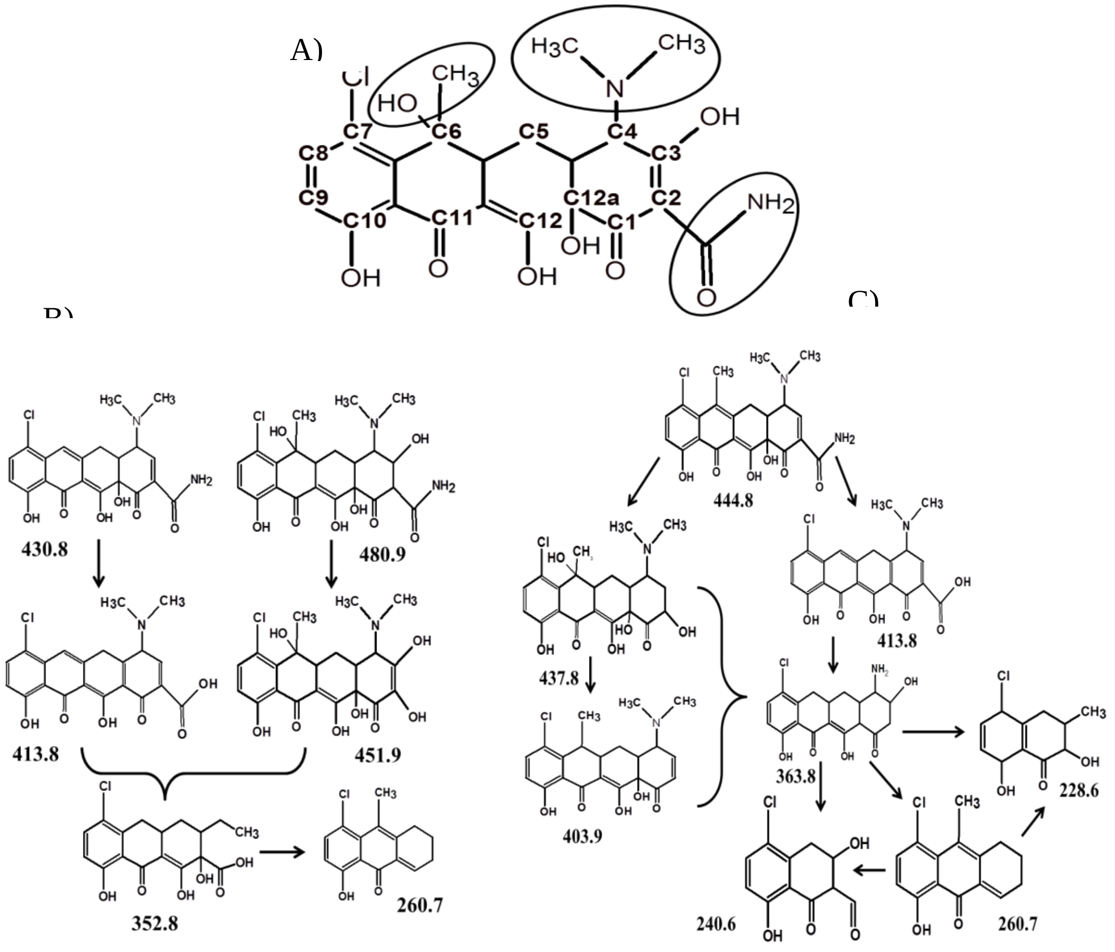


Figure 4.2.4 **Degradation pathways of CTC in wastewater; A) targeting sites of CTC; B) laccase degradation pathway of CTC;and C) ultrasonic assisted laccase degradation pathway.**

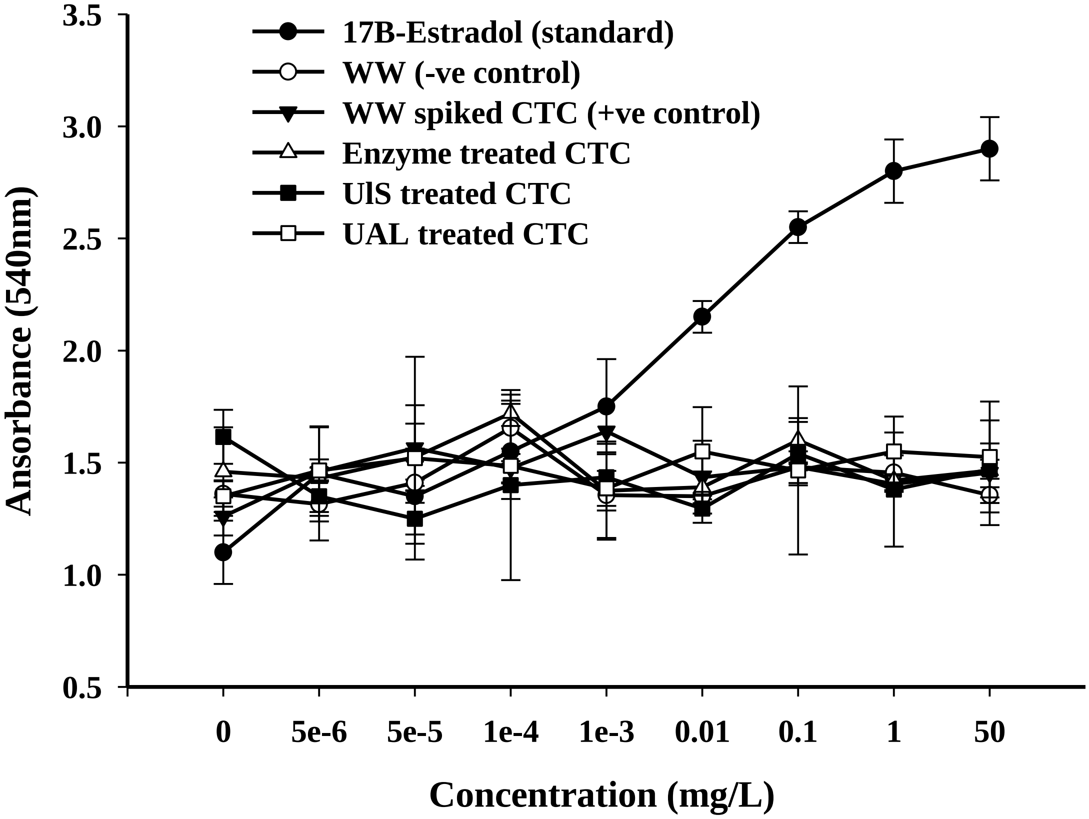


Figure 4.2.5 **Estrogenic response of treated wastewater spiked with chlortetracycline (CTC) using laccase, ultrasonication (UlS) and, ultrasonic assisted laccase (UAL) treatment.**

PART 3

ACTIVATION OF PERSULFATE BY HOMOGENOUS AND HETEROGENEOUS IRON CATALYST TO DEGRADE CHLORTETRACYCLINE IN AQUEOUS SOLUTION

Rama Pulicharla[a], Roggy Drouinaud[b], Satinder Kaur Brar[a]*, Patrick Drogui[a],
Mausam Verma[c], Rao Y. Surampalli[d]

[a] INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec, Canada G1K 9A9.

[b] Service du traitement des eaux, 214, Avenue St-Sacrement, suite 210, Québec, Canada G1N 3X6.

[c] CO2 Solutions Inc., 2300, Rue Jean-Perrin, Québec, Québec G2C 1T9 Canada.

[d] Department of Civil Engineering, University of Nebraska-Lincoln, N104 SEC PO Box 886105, Lincoln, NE 68588-6105, USA.

* Corresponding author: INRS-ETE, Téléphone: + 418 654 3116; Fax: + 418 654 2600, E-mail: satinder.brar@ete.inrs.ca;.

Résumé

La pseudo-persistance des antibiotiques de la tétracycline dans l'environnement pose un risque dû au développement de la résistance. Cette étude étudie l'élimination de l'antibiotique de chlortétracycline (CTC) en utilisant un procédé d'oxydation à base de radicaux sulfate. L'oxydation du CTC a été réalisée en utilisant du persulfate (PS) avec du fer dans des réactions homogènes (Fe2 +) et hétérogènes (Z-val Iron, ZVI). L'influence de divers paramètres, tels que l'effet de la posologie PS, la dose de fer, l'optimisation du rapport molaire PS / fer et l'effet du pH ont été étudiés et des conditions optimales ont été signalées. Les procédés PS / Fe2 + et PS / ZVI ont montré 76% et 94% de l'élimination de la CTC au rapport molaire 1: 2 pour 1 μM de CTC en 2h à 500 μM de PS et 1000 μM de fer. La dégradation de la CTC a augmenté avec un rapport molaire égal de PS / fer avec une concentration plus élevée de PS dans les processus PS/Fe2+ et PS/ZVI. Des doses de fer plus élevées ont montré une diminution de l'élimination du CTC dans le cas d'une réaction homogène (PS/Fe2+) à une concentration constante de PS de 500 μM alors que dans une réaction hétérogène (PS/ZVI), elle a montré une dégradation similaire avec une augmentation de la dose de fer. Au lieu de générer une production plus élevée de radicaux sulfate à la fois (PS/Fe2 +), la production lente dans le processus PS/ZVI semble plus efficace. Les produits de dégradation formés ont déclaré que les radicaux sulfate sont efficaces pour former des produits finis stables et non toxiques.

Mots clés: Chlortétracycline, Le fer, Complexation métallique, Persulfate, Radicaux sulfate.

Abstract

Pseudo-persistence of tetracycline antibiotics in the environment poses risk due to resistance development. This study investigates the removal of chlortetracycline (CTC) antibiotic using sulfate radical based advanced oxidation process. Oxidation of CTC was carried out by using persulfate (PS) with iron in homogeneous (Fe^{2+}) and heterogeneous (zero valent iron, ZVI) reactions. The influence of various parameters, such as effect of PS dosage, iron dosage, optimization of PS/iron molar ratio, and effect of pH were studied and optimum conditions were reported. PS/Fe^{2+} and PS/ZVI processes showed 76% and 94% of CTC removal at 1:2 molar ratio for 1 µM CTC in 2h at 500 µM PS and 1000 µM iron. CTC degradation increased with equal molar ratio of PS/iron with higher concentration of PS in both PS/Fe^{2+} and PS/ZVI processes. Higher iron dosages showed decreased CTC removal in the case of homogeneous reaction (PS/Fe^{2+}) at constant PS concentration 500 µM whereas in heterogeneous reaction (PS/ZVI), it showed similar degradation with increasing iron dose. Instead of generating higher production of sulfate radicals at a time (PS/Fe^{2+}), slow production in PS/ZVI process seems to be more efficient. Formed degradation products stated that sulfate radicals are efficient in forming stable and non-toxic end products.

Keywords: Chlortetracycline, Iron, Metal complexation, Persulfate, Sulfate radicals.

Introduction

Increased concentrations of contaminants in water sources demand their efficient removal to control pollution. For the last few decades, chemical oxidation, such as advanced oxidation processes (AOPs) has been competently used in treating contaminants in the natural environment by oxidants. AOPs including ozonation (Michael-Kordatou *et al.*, 2017), Fenton oxidation(Munoz *et al.*, 2016), ultrasonic oxidation(Mecha *et al.*, 2016), photo-Fenton(Miralles-Cuevas *et al.*, 2014), photocatalytic(Paul *et al.*, 2007, Qin *et al.*, 2016), and persulfate (PS)(Anipsitakis *et al.*, Lin *et al.*, 2016) processes have efficiently succeeded in dealing with recalcitrant contaminants. The emergence of different contaminants and to meet current environmental regulations, there has been a continuous drive towards technological advances in AOPs. Almost all AOPs comprise generation of hydroxyl radicals (OH^*) having a strong oxidation potential (E^0 = 2.7 V) and are non-selective towards most of the organic contaminants(Lee *et al.*, 2010). However, the oxidants used for generation of OH^*, such as ozone, hydrogen peroxide, and permanganate have higher solubility in water, higher reactivity and selective reactivity towards unsaturated compounds making them inefficient in treating recalcitrant contaminants, respectively. Further, these treatments alone showed limited ability to oxidize organic contaminants (Gogate *et al.*, 2004).

For advanced oxidation remediation technologies and to overcome the limitations of oxidants producing OH^*; PS oxidation has recently drawn much attention from researchers due to higher solubility and stability at room temperature and it also has high oxidation potential (E^0 = 2.01 V)(Liang *et al.*, 2004, Zhou *et al.*, 2013). Activation of PS produces high redox potential and non-selective sulfate radical (SO_4^{-*}, E^0 = 2.6 V) similar to OH^*(Vicente *et al.*, 2011). Moreover, $SO4^{-*}$ further induces hydroxyl radical's generation as per Eq. 4.

To generate $SO4^{-*}$, different approaches have been used to activate PS, such as increasing temperature(Luo, 2014), addition of transition metal ions(Anipsitakis *et al.*, 2004) and chelation agents(Liang *et al.*, 2009), ultraviolet irradiation(Lau *et al.*, 2007). Among these methods, iron (Fe) based metal activation was most studied and reported significantly enhanced SO_4^{-*} production rate(Monteagudo *et al.*, 2015, Oh *et al.*, 2009). Even iron-based PS activation involved both homogeneous (Fe^{2+}; soluble) to heterogeneous (Fe^0, insoluble) ways to activate persulfate(Gao *et al.*, 2016). And also while considering the features, cost-effectiveness, environmentally friendly nature, natural presence in the environment, and high activity, it is appropriate to develop sustainable remediation for contaminants using iron as a catalyst (Liang *et al.*, 2004, Romero *et al.*, 2010). Overall reactions involved in the process of generating $SO4^{-*}$ by activation of PS anion by ZVI and Fe^{2+} are described by Eq. 1-7(Liang *et al.*, 2007, McElroy *et al.*, 1990, Triszcz *et al.*, 2009, Wilmarth *et al.*, 1962):

$$S_2O_8^{2-} + Fe^{2+} \rightarrow Fe^{3+} + SO_4^{-*} \tag{1}$$

$$S_2O_8^{2-} + Fe^{2+} \rightarrow Fe^{3+} + SO_4^{2-} + SO_4^{-*} \tag{2}$$

$$SO_4^{-*} + Fe^{2+} \rightarrow Fe^{3+} + SO_4^{2-} \tag{3}$$

$$SO_4^{-*} + H_2O \rightarrow OH^* + H^+ + SO_4^{2-} \tag{4}$$

$$SO_4^{*-} + SO_4^{-*} \rightarrow 2SO_4^{2-} \tag{5}$$

$$SO_4^{-*} + OH^* \rightarrow HSO_4^{-} \tag{6}$$

$$S_2O_8^{2-} + Fe^0 \rightarrow Fe^{2+} + SO_4^{2-} \tag{7}$$

$$Fe^{3+} + Fe^0 \rightarrow Fe^{2+} \tag{8}$$

$$Fe^0 + H_2O \rightarrow Fe^{2+} + H_2 + OH^- \tag{9}$$

Tetracycline antibiotics are largely used in humans, veterinary, and aquaculture applications either to control diseases or for growth enhancement. Due to their high usage, researchers reported the omnipresence of tetracyclines as important contaminants in the environment detecting around 0.13−0.61 μg/L (Miao *et al.*, 2004, Puicharla *et al.*, 2014, Zhao *et al.*, 2010) in water. Chlortetracycline (CTC) is one of the old tetracycline family antibiotic, it has four-ring system with multiple O- and N-containing ionizable functional groups forms strong complexes with metals including iron(Pulicharla *et al.*, 2015, Wang *et al.*, 2015). Further, CTC-metal complexation in the aquatic environment is affecting its fate such as redox reactions and degradation. Fig. 1 shows the CTC structure with Fe^{2+} metal binding site. Until date, many oxidation technologies were developed to degrade chlortetracycline (CTC) antibiotic in the aqueous solution (Daghrir *et al.*, 2013, Daghrir *et al.*, 2012, Guo *et al.*, 2012, Hammad Khan *et al.*, 2013, Kim *et al.*, 2012). However, to our knowledge, PS/Fe^{2+} (homogeneous) and PS/ZVI (heterogeneous) processes have never been attempted to degrade CTC. In the case of PS/Fe^{2+} process, $SO4^{-*}$ can be rapidly scavenged by excess Fe^{2+} (Eq. 3), which affect the pollutant degradation efficiency(Liang *et al.*, 2004). Use of ZVI alternative to Fe^{2+} has been reported in the literature to overcome this disadvantage (Deng *et al.*, 2014, Liang *et al.*, 2010). Further, Fe^0 is able to produce and regenerate Fe^{2+} by reduction of PS (Eq. 7) and Fe^{3+} (Eq. 8), respectively.

The scope of this study is to evaluate the degradation efficiency of CTC by PS/Fe^{2+}/ZVI processes and specifically focuses on: 1) method development to estimate free CTC after complexation with iron using ultra high-pressure liquid chromatography-mass spectrometry (UHPLC-MS/MS); 2) PS/Fe^{2+} and PS/ZVI ratio optimization to produce sulfate radicals by PS activation to degrade CTC; 3) comparison of degradation efficiencies of CTC by PS/Fe^{2+} and PS/ZVI processes; 4) identification of degradation products and pathways; and 5) toxicity measurement of degraded CTC by determining estrogenicity.

Material and methods

Chemicals

CTC with 99% purity was purchased from Toronto Research Chemicals (Toronto, Canada). Disodium ethylenediaminetetraacetate (Na_2H_2EDTA, 99%) was purchased from E-bay (Tokyo, Japan). Methanol (HPLC grade, purity >99.8%), acetonitrile (ACN, UHPLC grade, purity >99.9%), formic acid (UHPLC grade, purity >99.9%) were purchased from Fisher Scientific (Ontario, Canada). Sodium persulfate (>98%), sodium azide (>99.9%), 1, 10-phenanthroline (>99%), 5-sulfosalicylic acid hydrate (>95%), potassium iodide (99%) and sodium bicarbonate (99.5%), were obtained from sigma-Aldrich (St. Louis, USA). Iron powder was purchased from Fisher scientific (Nazareth, USA). Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$; 99% analytical grade) was obtained from Fisher scientific (New Jersey, USA). pH was adjusted by using hydrochloric acid

(HCL, 99.9%, 5N) or sodium hydroxide (NaOH, 99.9%, 5M) (Merck, US). MilliQ/Reference with a LC-PAK polisher cartridge installed at the point-of-use and Milli-Q/Milli-RO Millipore systems (Milford, MA, USA) were used to prepare UHPLC and HPLC grade water in the laboratory.

Experimental procedure

Initial CTC degradation experiments were conducted for optimization of PS/Fe^{2+} and PS/ZVI processes. All the experiments were performed in 250 mL glass flasks with a total solution volume of 100 mL at 20±2 ^{o}C. PS/Fe^{2+} degradation experiments were carried out under constant stirring with a magnetic stirrer at 500 rpm and for PS/ZVI process, homogenizer was used to mix PS and ZVI for 2h. Concentrations of PS were taken based on the proposed stoichiometric Eq. 10. For the PS/Fe^{2+} and PS/ZVI processes, an initial ratio of 1:1 ratio was imposed with five different concentrations, including 100, 250, 500, 1000, 5000 µM by keeping constant CTC concentration of 1 µM, considering the PS optimization. Once the PS concentration was optimized based on the CTC removal efficiency, optimal PS to Fe^{2+} or ZVI ratio was investigated under various PS to Fe^{2+} or Fe^0 molar ratios, including 1:0.5, 1:1, 1:2, 1:3, and 1:4. After the ratio optimization of PS/Fe^{2+} and PS/ZVI processes, pH effect ranging from 3-10 was studied for CTC degradation. In all experiments, the reaction to produce SO_4^{-*} was quenched with sodium nitrite 0.1 M and further samples were filtered and stored at 4 ^{o}C, until CTC analysis. All experiments were carried out in triplicate and mean values of data was taken to present in all figures.

$$C_{22}H_{23}ClN_2O_8 + 48N_2S_2O_8 + 36H_2O \rightarrow 22CO_2 + 48Na_2SO_4 + 48H_2SO_4 + HCl + N_2 \qquad 10$$

Analytical methods

CTC estimation

Sample preparation for CTC analysis

All samples including standards and CTC experiments before and after degradation were filtered through acetone washed 2 mm glass fibers (Fisher brand G6 filter circles, Fisher Scientific, Ontario Canada). Sample preparation for CTC analysis followed the method of Pulicharla et al., (Puicharla *et al.*, 2014) with small modifications. EDTA concentration was optimized in this study to release the CTC from CTC-Fe complexation and get maximum CTC recovery. About 5 mL of filtrate was taken and EDTA of 1-10 mM was added before adjusting the pH with 5 N HCl to 2.7–3. After pH adjustment, samples were immediately subjected to clean up with Sep-pack® C18 Plus Short Cartridges. Cartridges were fixed in a vacuum manifold (Welch, USA) connected to a vacuum pump (Welch Rietschle Thomas, USA) for extracting CTC. Before extracting samples, cartridges were preconditioned with 5 mL of methanol and 5 mL of milli-Q water with a flow rate of approximately 1 mL/min. Samples were loaded into the cartridges and extracted approximately 1.5 mL/min flow rate. After extracting the sample, the cartridges were dried using the vacuum system set at 15 psi for 10 min. Finally, CTC was eluted with equal volume 5 mL of methanol at a flow rate of 1 mL/min in order to give enough time for methanol to elute adsorbed CTC (Puicharla *et al.*, 2014). This final extraction was stored at 4 ^{o}C until injecting into UHPLC. Loss of CTC during extraction was determined by spiking CTC milli-Q water and for the matrix effects (Fe^{2+}, ZVI, and PS) determination. CTC was spiked in 5 mL milli-Q water containing respective matrix before extraction. The CTC was measured with the UHPLC–MS/MS method developed in this study and spike recoveries were calculated.

UPLC–MS/MS

CTC was analyzed in the extraction samples by Waters Acquity I-Class (Milford, USA) UHPLC system with an Agilent Zorbax SB-C18 RRHD (Santa Clara, USA) reverse-phase column (2.1 × 50 mm, 1.8 μm). UHPLC–ESI (electrospray ionization) source mounted on a Xevo TQ-S Mass Spectrometer (Waters, Milford, USA) was used for CTC quantification. Mobile phase, solvent A (0.4% formic acid) and solvent B (acetonitrile) was optimized in a gradient elution as follows: 77.5% A (0-1.5 min), 10% A (1.51-2.5 min), 77.5% A (2.51-3 min) at acidic pH (2.7±0.5). Other parameters such as column temperature (30 ^{o}C), injection volume (1 μL) and flow rate (0.45 mL/min) were optimized for CTC method quantification in UHPLC. The mass spectrometer (MS/MS) was operated in positive ion mode and multiple reaction transitions (MRM) were used to monitor the CTC acquisition. The optimized MS/MS parameters in MRM with the highest intensity for CTC transitions including their precursor and product ions, cone voltage and collision energy are summarized in table 4.3.1. The product ion 154 producing the highest intensity CTC transition was used for quantitation and 444 transitions were used for confirmation of CTC. Other instrumental parameters used were as follows: spray voltage, 4.0 kV; collision gas, Argon at 0.15 ml/min; source temperature, 150 °C; desolvation temperature, gas flow, and pressure were 650°C, 1200 L/h and 5.0 bar respectively; cone gas flow, 150 L/h . The five-point calibration curve (5 µg/L to 200 µg/L) was prepared from a stock solution of 500 µg/L CTC in water.

CTC transformation products were analyzed in a reverse-phase Hypersil Gold C18 column (100 x 2.1mm, 3µm particle size) by LC-MS/MS (Thermo fisher scientific) coupled to a 4000 TSQ Quantum Access Mass Spectrometer equipped with an electrospray ionization source. The mobile phase used for determination of CTC byproducts consists of 0.1% acetic acid in MilliQ water and 0.1% acetic acid in acetonitrile at a flow rate of 230 µL/min, 20 µl injection volume and column temperature of 30 °C. Positive electrospray ionization of mass spectrometric analysis was conducted with a mass scan range of m/z 50−500.

Persulfate determination

Production of $SO4^{-*}$ was indirectly measured by determining the persulfate concentration consumed during the experiment. A rapid spectrophotometric iodometric method of persulfate anion determination developed by Chenju Liang et al., (Liang *et al.*, 2008) was used to measure the unused persulfate left in the solution after 2h of experimental time. The reaction mixture containing, sodium persulfate stock solution/treated samples, $NaHCO_3$ and KI in Milli-Q water in glass vials were hand shaken and allowed to equilibrate for 15 min. Yellow iodine color formed from the reaction of PS with KI as shown in Eq. 11 was measured using a UV spectrophotometer at 352 nm. Experiments mean data and standard deviation is presented in all figures.

$$S2O8^{2-} + 2I^{-} \rightarrow 2SO_4^{2-} + I_2 \qquad 11$$

Fe^{2+} and Fe^{3+} analysis

A spectrophotometric method was used to determine the Fe^{2+} and Fe^{3+} concentrations separately. 1,10-phenanthroline and sulfosalicylic acid indicator reagents were used to form complexes with Fe^{2+} (3:1, λ = 512 nm) and Fe^{3+} (1:1, λ = 490 nm) and signal was measured respectively, in acid environment (pH ≈3)(Kozak *et al.*, 2010). The absorbance was measured on a Varian Carey 50 UV/visible spectrophotometer. A Calibration curve of known $FeSO_4.7H_2O$ concentrations was used to calculate the Fe^{2+} concentrations. These reagents are known to form stable complexes with respective iron ions in the acidic environment(Oktavia *et al.*, 2008). Furthermore, reagents concentration used in this study were sufficient to fully outcompete CTC, resulting in negligible free Fe^{2+} and Fe^{3+} and CTC-Fe complexes.

Yeast Estrogen Screen (YES) assay

In order to screen the estrogenicity of the treated CTC, YES assay was carried out. This test was carried out according to Routledge and Sumpter (1996) (Routledge *et al.*, 1996) studies to measure the estrogenic activity of PS/Fe^{2+} and PS/ZVI systems degraded CTC. Sterilization of all samples and standard (CTC and 17β-estradiol) was carried out prior to YES assay by serial dilutions with ethanol. Each concentration of standard and samples of 10 µL aliquots were transferred (triplicate) to a 96-well plate (Costar Brand, NY, USA) and waiting until all samples were completely evaporated at room temperature. All experiments were conducted in the laminar flow chamber. Further, aliquots (200 µL) of seeded assay medium containing the recombinant yeast (hER-transfected recombinant yeast) and the chlorophenol red-β-d-galactopyranoside (CPRG) chromogenic substrate were dispensed into each sample well. Each plate contained a row of an appropriate solvent controls (assay medium and pure ethanol) and a standard curve for 17β-estradiol. The plates were sealed with autoclave tape and mixed vigorously for 10 min in a shaker for 5min and incubated for 3 days at 32°C. The color of the medium was read after 3 days of incubation using a multireader microplate spectrophotometer (Epoch, BioTek, USA) at an absorbance of 540 nm.

Statistical analysis

Mean ± standard deviation was used to summarize the data in this study. Effect of PS/Fe^{2+} and PS/ZVI processes on the degradation of CTC data was statistically evaluated using analysis of variance (ANOVA, SigmaPlot). Data from triplicates of CTC removal efficiency was expressed as the mean± standard error.

Results and discussion

CTC estimation

It is worth underlining that when analyzing CTC spiked in MilliQ water in the presence of Fe^{2+} and Fe^0 using a previous LC-Ms method developed by Daghrir et al., (Daghrir *et al.*, 2014), it was observed that less than 10% recovery of CTC complexed with iron. It has to be pointed out that this is not the drawback of the method as CTC forms stable complexes with Fe^{2+} (Wang *et al.*, 2015)and this method is not able to break the CTC-Fe complex to measure CTC. Hence, small modifications were done in the method and CTC was measured using UHPLC-MS/MS. It was also observed that basic pH mobile phase (0.1% NH_4OH and ACN) is unable to give good recovery (15%) for CTC. During method development, direct injection of CTC solution containing iron after 30 min interaction between CTC and iron has given 77% recovery where after the overnight interaction has shown less than 10% CTC recovery (presented as supplementary data).

In the current work, solid phase extraction (SPE) was used to break the CTC-Fe complex and extract CTC with additional small modifications to previous studies of Pulicharla et al. 2014 (Puicharla *et al.*, 2014). Added EDTA (1-10 mM) concentration to release CTC from metal complexes has been optimized and 3 mM EDTA was found to be optimum for the complexed 1 µM CTC. Further, a mobile phase having pH 2.7±0.5 with 0.4% formic acid and ACN in gradient elution as mentioned in session to 2.3.1.2 showed 95% recovery of CTC. The recoveries of CTC were measured by extracting analytes from 5 mL of MilliQ water spiked at 1 µM CTC before and after treatments. Following Eq. 12 was applied to calculate the recoveries of all samples.

$$\%\,recovery = (C_f/C_i) \times 100 \qquad 12$$

where C_f = measured concentration of CTC after extraction, C_i = initial concentration of CTC spiked in sample. Recoveries of spiked CTC at 1 µM in deionized water were found to be 93–105%. This indicates the complete release of CTC from its iron metal complex with the developed method. The calibration curve with five concentration levels (5–200 µg/L) showed good linearity with a correlation coefficient (R^2) of 0.995. Method validation parameters developed in this study have been summarized in table 4.3.2.

Degradation of chlortetracycline by sulfate radicals

Figure 4.3.2 shows the CTC removal efficiency of PS, Fe^{2+} and ZVI systems alone as positive controls. The removal efficiency was lower at low concentrations of PS, $Fe^{2+,}$ and ZVI and maximum removal of 54%, 44% and 28% CTC was obtained at a highest concentration 5000 µM of respective controls studied in the current study.

Even though PS is stable and did not produce any sulfate radicals at ambient temperature, high oxidation potential (E^0 = 2.01 V) was able to degrade CTC with increasing PS concentration. In the case of Fe^{2+} which is in homogeneous phase as compared to CTC, Fe^{2+} is able to form stable complexes with CTC and undergo autoxidation from Fe^{2+} to Fe^{3+}. This autoxidation was enhanced in the presence of CTC and also simultaneous CTC degradation was reported by Wang et al., 2016 (Wang *et al.*, 2015). This study reported the production of radicals such as O_2^{-*}, H_2O_2, OH^* during autoxidation of Fe^{2+} which are responsible for the CTC degradation. Similar results were obtained in this study, where increased iron concentration showed increased CTC degradation. Low CTC degradation in ZVI compared to Fe^{2+} was due to low availability of Fe^{2+} to complex and degrade CTC and also slow oxidation of Fe^0 to generate Fe^{2+} at room temperature (Gomathi Devi *et al.*, 2009). Only 10 -15% of Fe^{2+} was formed in all experiments of ZVI after 2h at room temperature (data not shown).

Optimization of PS/Fe^{2+} and PS/ZVI processes

Optimization studies were performed by taking the equal molar ratio of PS/Fe^{2+} and PS/ZVI. Based on the stoichiometric Eq. 10, 48 times molar PS concentration is needed to completely mineralize one molar CTC. Taking this into consideration, five different molar ratios ranging from 100-5000 µM of PS was studied. Figure 4.3.3 represents the comparison of CTC degradation efficiency by Fe^{2+} and ZVI activating persulfate at equal molar ratios. With the increasing molar ratios, increased removal efficiency was seen in both PS/Fe^{2+} and PS/ZVI processes. Highest CTC removal of 87% and 94% was obtained in PS/Fe^{2+} and PS/ZVI processes, respectively at highest molar ratios. In control experiments, it was confirmed that PS alone can degrade 54% of CTC at its highest molar concentration. CTC degradation was increased to a large extent when PS was combined with Fe^{2+} and ZVI at room temperature, (Figure 4.3.3). It can be explained that formation of $SO4^{-*}$ by activation of PS by Fe^{2+} and ZVI at room temperature might have helped in the degradation process. Figure 4.3.2 clearly indicates that PS is activated by Fe^{2+} and ZVI with different efficiencies which resulted in different CTC removal. When PS is activated, 50-60% and 35-47% of PS was consumed for all ratios of PS/Fe^{2+} and PS/ZVI, respectively to form $SO4^{-*}$. Even though the activation is similar for all ratios of PS/Fe^{2+} and PS/ZVI, still the amount of sulfate radicals formed increased with increasing ratios and thus increased CTC removal. Instead of using a high concentration of PS to degrade CTC, iron concentration which acts as a catalyst to produce $SO4^{-*}$ has been optimized for maximum activation of PS and CTC removal.

For further iron optimization, 500 µM PS was taken as constant where 50% and 35% of PS was activated and 66% and 72% of CTC was removed with an equal concentration of Fe^{2+} and ZVI, respectively (Figure 4.3.2). Five different ratios of Fe^{2+} and ZVI with respect to PS were tested for iron optimization. Figure 4.3.4(A) shows the optimized iron concentration for maximum CTC removal. Two times the PS concentration of iron (1:2) showed 77%±3.4 and 93%±2.9 CTC removal in both PS/Fe^{2+} and PS/ZVI processes, respectively. Figure 4.3.4(B) indirectly represents the formation of sulfate radicals with % of PS that is activated. 91%±2.3 and 53%±1.8 of PS has been activated to remove CTC in PS/Fe^{2+} process and PS/ZVI process respectively. Even though PS consumption is high (91%) in PS/Fe^{2+} process, high CTC removal was observed in PS/ZVI process at 53% PS activation.

In case of PS/ZVI system, the release of Fe^{2+} from Fe^0 and further oxidation of Fe^{2+} to Fe^{3+} were the two principal reactions involved in the activation of PS. This continuous release and supply of Fe^{2+} from heterogeneous phase might activate PS efficiently than a one-time dose of Fe^{2+} in PS/Fe^{2+} homogeneous system. Apart from this, at 1:2 (PS/Fe^{2+}) ratio, above 90% of PS has been activated but CTC removal was increased by only 10% (77%) compared to 1:1 molar ratio. This can explain the fact that higher generation of SO_4^{-*} might act as scavenger itself as given in Eq. 5(Xu *et al.*, 2010) and also the higher Fe^{2+} concentration can also scavenge SO_4^{-*} as given in Eq. 3(McElroy *et al.*, 1990). Many studies have reported that Fe^{2+} oxidant molar ratio over 1:1 to PS was not efficient in oxidizing organic contaminants as generated SO_4^{-*} are getting scavenged by other side reactions (Brandt *et al.*, 1995, Rastogi *et al.*, 2009). In contrast, only half activated PS (53%) results in 93% CTC removal by ZVI at 1:2 (PS/ZVI). Therefore, as compared to the CTC removal by homogeneous availability of Fe^{2+} activated PS (Figure 4.3.3), the extent of CTC removal by heterogeneous ZVI activated PS was efficient under identical conditions. These results indicated that the removal efficiency of CTC could be enhanced by ZVI instead of Fe^{2+} addition for PS activation. Further, the estimation of Fe^{2+} concentration after 2h reaction evidenced that slow activation of PS is effective in CTC degradation. Above 80% of total Fe^{2+} was oxidized to Fe^{3+} in case of PS/Fe^{2+} (1:2) where in PS/ZVI reaction 95% of dissolved Fe^{2+} that was released after 2h from ZVI (25-30%) was oxidized to Fe^{3+}. Further, low Fe^{3+} in PS/ZVI (1:2) was seen at the end compare to PS/Fe^{2+} reaction which is due to less available concentration of Fe^{2+} undergone oxidation to Fe^{3+}. Additional kinetic studies are necessary to evaluate the oxidation rate of Fe^{2+} and its regeneration.

Effect of pH on CTC degradation

Optimum conditions of PS/Fe^{2+} and PS/ZVI processes (1:2) where highest CTC removal was observed have been used to investigate the pH effect on CTC degradation. Five different pH values including 3.0, 4.5, 6.0, 8.0 and 10.0 were studied and pH adjustment was done using NaOH or HCl. As can be seen in figure 4.3.5, CTC removal was decreased with the increased pH. Final pH after the 2h reaction was recorded as follows 2.68, 2.66, 2.8, 3.1 and 3.5 for respective studied initial pHs. CTC removal efficiency was nearly the same until pH 8 and up to 15% decrease in removal was seen until pH 10. As reported, SO_4^{-*} can be produced at a wide range of pH (2-8), the decreased removal can be due to scavenging of SO_4^{-*} by OH^* at higher pH (Eq. 6)(Liang *et al.*, 2007). At the same time, it was reported that SO_4^{-*} can generate OH^* under the alkaline condition as shown in Eq. 4 (Wu, 2008). However, the OH^* radicals oxidation potential may decrease with increasing pH.

The final removal efficiency was 75%, 76%, 74%, 69% and 62% for Fe^{2+} and 94%, 92%, 92%, 88% and 78% for ZVI at pH 3.0, 4.5, 6.0, 8.0 and 10.0, respectively as shown in figure 4.3.4. In this study, pH is not maintained throughout the study by adding any buffer solution, only initial pH effect was investigated to avoid unnecessary interactions among buffer reagents, SO_4^{-*} radicals, and CTC. From these results, it is very clear that the CTC degradation efficiency is not affected by the initial pH in both systems from pH 3.0–8.0. These results are in agreement with the previous studies of Hou et al. (Hou *et al.*, 2012). Sulfuric acid is produced during the reaction as seen in the Eq. 10. Therefore, this could be the reason for the drop of pH during the reaction. Nearly similar final pH value at the end of all experiments stated the similar degradation efficiency despite different initial pH values.

Identification of degradation products and proposed pathways

After the maximum removal of CTC achieved in PS/Fe^{2+} and PS/ZVI processes, the solutions were analyzed by LC-MS/MS. According to the MS spectrometric results, the possible mechanisms through which sulfate radicals degraded CTC under PS/Fe^{2+} and PS/ZVI processes is proposed in figure 4.3.6. The intermediates formed during the degradation process were identified and reported for the first time by sulfate radicals in this study. Both processes have shown similar TPs, such as m/z 303 and 184 with highest intensities compared to parent compound. Both processes attacked CTC in similar fashion by demethylation, deamination dehydroxylation reactions, but the formed end degradation products were dissimilar. This might be due to higher efficiency of PS/ZVI process which might degrade CTC to lower mass TPs compared to PS/Fe^{2+} system. And also the mechanism of CTC degradation by sulfate radicals is similar to hydroxyl radical (Halling-Sørensen *et al.*, 2002, Pulicharla *et al.*, 2017). However, on comparing the degradation products, sulfate radicals seem to be more efficient in degrading CTC. Formation of lower mass and dechlorinated TPs in this study showed that sulfate radicals are generally efficient in degrading organic contaminants having chlorine in their structure.

Estrogenicity assessment of PS/Fe^{2+} and PS/ZVI treated CTC samples

Estrogen activity of experiments (1:2; PS/Fe^{2+} and PS/ZVI processes) which showed highest CTC removal was carried out by YES assay. The response of estrogenic activity of standard 17β-estradiol (5 ng/L to 50mg/L) concentrations showed absorbance within 1 to 1.2 range after three days incubation. CTC spiked in MilliQ water before and after treatment with PS/Fe^{2+} and PS/ZVI processes has shown no estrogenic activity compared to tested standard 17β-estradiol standard samples. Assay medium treated with standard 17β-estradiol only showed red color and remaining samples including blank (alcohol), spiked and treated CTC samples did not show any color change to red. The different TPs of both PS/Fe^{2+} and PS/ZVI processes (Figure 4.3.5) which are not completely similar did not show any estrogenicity. Controls having Fe^{2+}, ZVI and PS samples also did not have estrogenic activity. Hence, the used PS and iron concentrations in this study 500 µM and 1000 µM were not toxic respectively. As per author's knowledge, this is the first time reporting the estrogenic activity of PS and iron concentrations and also the CTC degradation by SO_4^{-*}. Similar to this study, there was no estrogenic activity resulting from CTC TPs by OH^* (Pulicharla *et al.*, 2017) Hence, SO_4^{-*} can be considered safe and efficient in degrading CTC.

Conclusions

Free CTC after complexation with iron was measured with 95% recovery in currently developed UHPLC method. SPE with optimized EDTA (3mM) is necessary to break the CTC-Fe complex before analysis. In the present study, CTC was successfully degraded by environmental friendly SO4-* based AOP and it was found that heterogeneous activation (ZVI) is more efficient than homogeneous activation (Fe2+) of PS to degrade CTC. PS/Fe2+ and PS/ZVI processes showed 76 and 94% degradation at 1:2 molar ratio as optimum for degrading 1 µM CTC in 2h at 500 µM PS and 1000 µM iron. All Fe2+ ions in the case of homogeneous reaction activated above 90% PS to produce SO4-* which seemed to be not efficient in degrading CTC due to scavenging of sulfate radicals by it and also presence of excess Fe2+ ions. Whereas in heterogeneous reactions, slow generation and regeneration of Fe2+ ions might be helping in removal of CTC above 90%. Hence, heterogeneous activation by iron catalyst which is not in the same dissolved phase to PS demonstrated superior performance in CTC removal and in forming TPs than homogeneous activation under similar conditions. Further, observing dechlorinated end products stated that SO4-* is most effective in degrading organic contaminants to non-toxic compounds as none of the products demonstrated estrogenic effects.

Acknowledgement

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Discovery Grant 355254 and Strategic Grant), and Ministère des Relations Internationales du Québec (coopération Québec-Catalanya 2012–2014, 01.801-23525) for financial support. Authors are also thankful to “merit scholarship program for foreign students” (FQRNT) for financial assistance to Rama Pulicharla. Thanks to Prof. Viviane Yargeau for providing YES test yeast strain from McGill University to perform estrogenicity study.

References

Anipsitakis GP & Dionysiou DD (2004) Radical generation by the interaction of transition metals with common oxidants. *Environmental science & technology* 38(13):3705-3712.

Brandt C & Van Eldik R (1995) Transition metal-catalyzed oxidation of sulfur (IV) oxides. Atmospheric-relevant processes and mechanisms. *Chemical Reviews* 95(1):119-190.

Daghrir R, Drogui P, Delegan N & El Khakani MA (2013) Electrochemical degradation of chlortetracycline using N-doped Ti/TiO 2 photoanode under sunlight irradiations. *Water research* 47(17):6801-6810.

Daghrir R, Drogui P, Delegan N & El Khakani MA (2014) Removal of chlortetracycline from spiked municipal wastewater using a photoelectrocatalytic process operated under sunlight irradiations. *Science of The Total Environment* 466–467:300-305.

Daghrir R, Drogui P, Ka I & El Khakani MA (2012) Photoelectrocatalytic degradation of chlortetracycline using Ti/TiO 2 nanostructured electrodes deposited by means of a pulsed laser deposition process. *Journal of hazardous materials* 199:15-24.

Deng J, Shao Y, Gao N, Deng Y, Tan C & Zhou S (2014) Zero-valent iron/persulfate (Fe0/PS) oxidation acetaminophen in water. *International Journal of Environmental Science and Technology* 11(4):881-890.

Gao Y, Zhang Z, Li S, Liu J, Yao L, Li Y & Zhang H (2016) Insights into the mechanism of heterogeneous activation of persulfate with a clay/iron-based catalyst under visible LED light irradiation. *Applied Catalysis B: Environmental* 185:22-30.

Gogate PR & Pandit AB (2004) A review of imperative technologies for wastewater treatment I: oxidation technologies at ambient conditions. *Advances in Environmental Research* 8(3):501-551.

Gomathi Devi L, Girish Kumar S, Mohan Reddy K & Munikrishnappa C (2009) Photo degradation of Methyl Orange an azo dye by Advanced Fenton Process using zero valent metallic iron: Influence of various reaction parameters and its degradation mechanism. *Journal of Hazardous Materials* 164(2–3):459-467.

Guo R & Chen J (2012) Phytoplankton toxicity of the antibiotic chlortetracycline and its UV light degradation products. *Chemosphere* 87(11):1254-1259.

Halling-Sørensen B, Sengeløv G & Tjørnelund J (2002) Toxicity of tetracyclines and tetracycline degradation products to environmentally relevant bacteria, including selected tetracycline-resistant bacteria. *Archives of Environmental Contamination and Toxicology* 42(3):263-271.

Hammad Khan M, Jung H-S, Lee W & Jung J-Y (2013) Chlortetracycline degradation by photocatalytic ozonation in the aqueous phase: mineralization and the effects on biodegradability. *Environmental technology* 34(4):495-502.

Hou L, Zhang H & Xue X (2012) Ultrasound enhanced heterogeneous activation of peroxydisulfate by magnetite catalyst for the degradation of tetracycline in water. *Separation and Purification Technology* 84:147-152.

Kim T-H, Kim SD, Kim HY, Lim SJ, Lee M & Yu S (2012) Degradation and toxicity assessment of sulfamethoxazole and chlortetracycline using electron beam, ozone and UV. *Journal of hazardous materials* 227:237-242.

Kozak J, Gutowski J, Kozak M, Wieczorek M & Kościelniak P (2010) New method for simultaneous determination of Fe (II) and Fe (III) in water using flow injection technique. *Analytica chimica acta* 668(1):8-12.

Lau TK, Chu W & Graham NJ (2007) The aqueous degradation of butylated hydroxyanisole by UV/S2O82-: study of reaction mechanisms via dimerization and mineralization. *Environmental science & technology* 41(2):613-619.

Lee Y & von Gunten U (2010) Oxidative transformation of micropollutants during municipal wastewater treatment: comparison of kinetic aspects of selective (chlorine, chlorine dioxide, ferrate VI, and ozone) and non-selective oxidants (hydroxyl radical). *Water Research* 44(2):555-566.

Liang C, Bruell CJ, Marley MC & Sperry KL (2004) Persulfate oxidation for in situ remediation of TCE. I. Activated by ferrous ion with and without a persulfate–thiosulfate redox couple. *Chemosphere* 55(9):1213-1223.

Liang C & Guo Y-y (2010) Mass transfer and chemical oxidation of naphthalene particles with zerovalent iron activated persulfate. *Environmental science & technology* 44(21):8203-8208.

Liang C, Huang C-F, Mohanty N & Kurakalva RM (2008) A rapid spectrophotometric determination of persulfate anion in ISCO. *Chemosphere* 73(9):1540-1543.

Liang C, Liang C-P & Chen C-C (2009) pH dependence of persulfate activation by EDTA/Fe (III) for degradation of trichloroethylene. *Journal of contaminant hydrology* 106(3):173-182.

Liang C, Wang Z-S & Bruell CJ (2007) Influence of pH on persulfate oxidation of TCE at ambient temperatures. *Chemosphere* 66(1):106-113.

Lin Y-T, Liang C & Yu C-W (2016) Trichloroethylene degradation by various forms of iron activated persulfate oxidation with or without the assistance of ascorbic acid. *Industrial & Engineering Chemistry Research* 55(8):2302-2308.

Luo Q (2014) Oxidative treatment of aqueous monochlorobenzene with thermally-activated persulfate. *Frontiers of Environmental Science & Engineering* 8(2):188-194.

McElroy W & Waygood S (1990) Kinetics of the reactions of the SO–4 radical with SO–4, S2O2–8, H2O and Fe2+. *Journal of the Chemical Society, Faraday Transactions* 86(14):2557-2564.

Mecha AC, Onyango MS, Ochieng A, Fourie CJS & Momba MNB (2016) Synergistic effect of UV–vis and solar photocatalytic ozonation on the degradation of phenol in municipal wastewater: A comparative study. *Journal of Catalysis* 341:116-125.

Miao X-S, Bishay F, Chen M & Metcalfe CD (2004) Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. *Environmental science & technology* 38(13):3533-3541.

Michael-Kordatou I, Andreou R, Iacovou M, Frontistis Z, Hapeshi E, Michael C & Fatta-Kassinos D (2017) On the capacity of ozonation to remove antimicrobial compounds, resistant bacteria and toxicity from urban wastewater effluents. *Journal of Hazardous Materials* 323, Part A:414-425.

Miralles-Cuevas S, Oller I, Pérez JAS & Malato S (2014) Removal of pharmaceuticals from MWTP effluent by nanofiltration and solar photo-Fenton using two different iron complexes at neutral pH. *Water Research* 64:23-31.

Monteagudo J, Durán A, González R & Expósito A (2015) In situ chemical oxidation of carbamazepine solutions using persulfate simultaneously activated by heat energy, UV light, Fe 2+ ions, and H 2 O 2. *Applied Catalysis B: Environmental* 176:120-129.

Munoz M, Garcia-Muñoz P, Pliego G, Pedro ZMd, Zazo JA, Casas JA & Rodriguez JJ (2016) Application of intensified Fenton oxidation to the treatment of hospital wastewater: Kinetics, ecotoxicity and disinfection. *Journal of Environmental Chemical Engineering* 4(4, Part A):4107-4112.

Oh S-Y, Kim H-W, Park J-M, Park H-S & Yoon C (2009) Oxidation of polyvinyl alcohol by persulfate activated with heat, Fe 2+, and zero-valent iron. *Journal of hazardous materials* 168(1):346-351.

Oktavia B, Lim LW & Takeuchi T (2008) Simultaneous determination of Fe (III) and Fe (II) ions via complexation with salicylic acid and 1, 10-phenanthroline in microcolumn ion chromatography. *Analytical Sciences* 24(11):1487-1492.

Paul T, Miller PL & Strathmann TJ (2007) Visible-light-mediated TiO2 photocatalysis of fluoroquinolone antibacterial agents. *Environmental science & technology* 41(13):4720-4727.

Puicharla R, Mohapatra D, Brar S, Drogui P, Auger S & Surampalli R (2014) A persistent antibiotic partitioning and co-relation with metals in wastewater treatment plant—chlortetracycline. *Journal of Environmental Chemical Engineering* 2(3):1596-1603.

Pulicharla R, Brar SK, Rouissi T, Auger S, Drogui P, Verma M & Surampalli RY (2017) Degradation of chlortetracycline in wastewater sludge by ultrasonication, Fenton oxidation, and ferro-sonication. *Ultrasonics Sonochemistry* 34:332-342.

Pulicharla R, Das RK, Brar SK, Drogui P, Sarma SJ, Verma M, Surampalli RY & Valero JR (2015) Toxicity of chlortetracycline and its metal complexes to model microorganisms in wastewater sludge. *Science of the Total Environment* 532:669-675.

Qin D, Lu W, Wang X, Li N, Chen X, Zhu Z & Chen W (2016) Graphitic Carbon Nitride from Burial to Re-emergence on Polyethylene Terephthalate Nanofibers as an Easily Recycled Photocatalyst for Degrading Antibiotics under Solar Irradiation. *ACS Applied Materials & Interfaces* 8(39):25962-25970.

Rastogi A, Al-Abed SR & Dionysiou DD (2009) Sulfate radical-based ferrous–peroxymonosulfate oxidative system for PCBs degradation in aqueous and sediment systems. *Applied Catalysis B: Environmental* 85(3–4):171-179.

Romero A, Santos A, Vicente F & González C (2010) Diuron abatement using activated persulphate: effect of pH, Fe (II) and oxidant dosage. *Chemical Engineering Journal* 162(1):257-265.

Routledge EJ & Sumpter JP (1996) Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environmental toxicology and chemistry* 15(3):241-248.

Triszcz JM, Porta A & Einschlag FSG (2009) Effect of operating conditions on iron corrosion rates in zero-valent iron systems for arsenic removal. *Chemical Engineering Journal* 150(2):431-439.

Vicente F, Santos A, Romero A & Rodriguez S (2011) Kinetic study of diuron oxidation and mineralization by persulphate: effects of temperature, oxidant concentration and iron dosage method. *Chemical engineering journal* 170(1):127-135.

Wang H, Yao H, Sun P, Li D & Huang C-H (2015) Transformation of tetracycline antibiotics and Fe (II) and Fe (III) species induced by their complexation. *Environmental science & technology* 50(1):145-153.

Watkinson A, Murby E, Kolpin D & Costanzo S (2009) The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. *Science of the total environment* 407(8):2711-2723.

Wilmarth W, Haim A & Edwards J (1962) Mechanisms of Oxidation by Peroxydisulfate Ion in Peroxide Reaction Mechanisms. (Wiley-Interscience, New York).

Wu C-H (2008) Effects of operational parameters on the decolorization of CI Reactive Red 198 in UV/TiO 2-based systems. *Dyes and Pigments* 77(1):31-38.

Xu X-R & Li X-Z (2010) Degradation of azo dye Orange G in aqueous solutions by persulfate with ferrous ion. *Separation and purification technology* 72(1):105-111.

Zhao L, Dong YH & Wang H (2010) Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. *Science of the Total Environment* 408(5):1069-1075.

Zhou L, Zheng W, Ji Y, Zhang J, Zeng C, Zhang Y, Wang Q & Yang X (2013) Ferrous-activated persulfate oxidation of arsenic (III) and diuron in aquatic system. *Journal of hazardous materials* 263:422-430.

Table 4.3.1 Precursor and product ions, cone voltage and collision energy of the investigated antibiotic chlortetracycline (CTC)

Compound	**Precursor ion mass (Da) $[M+H]^+$ (m/z)**	**Product ion mass (Da)**	**Cone voltage (V)**	**Collision energy (V)**
Chlortetracycline	478.9	154.0	24	24
	478.9	444.0	24	16

Table 4.3.2. Method validation parameters of chlortetracycline (CTC) in UHPLC-MS/MS

	R^2	**Intra-day precision (%RSD)**	**Inter-day precision (%RSD)**	**IDL (μ/L)**	**MDL (μ/L)**	
					LOD	**LOQ**
MilliQ water	0.995	2.67	6.75	0.6	1.2	3.5

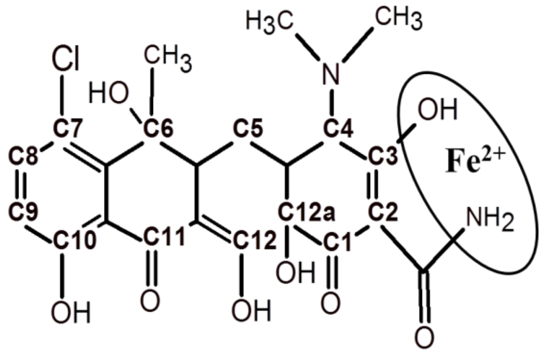


Figure 4.3.1 Chlortetracycline structure showing the iron binding cite.

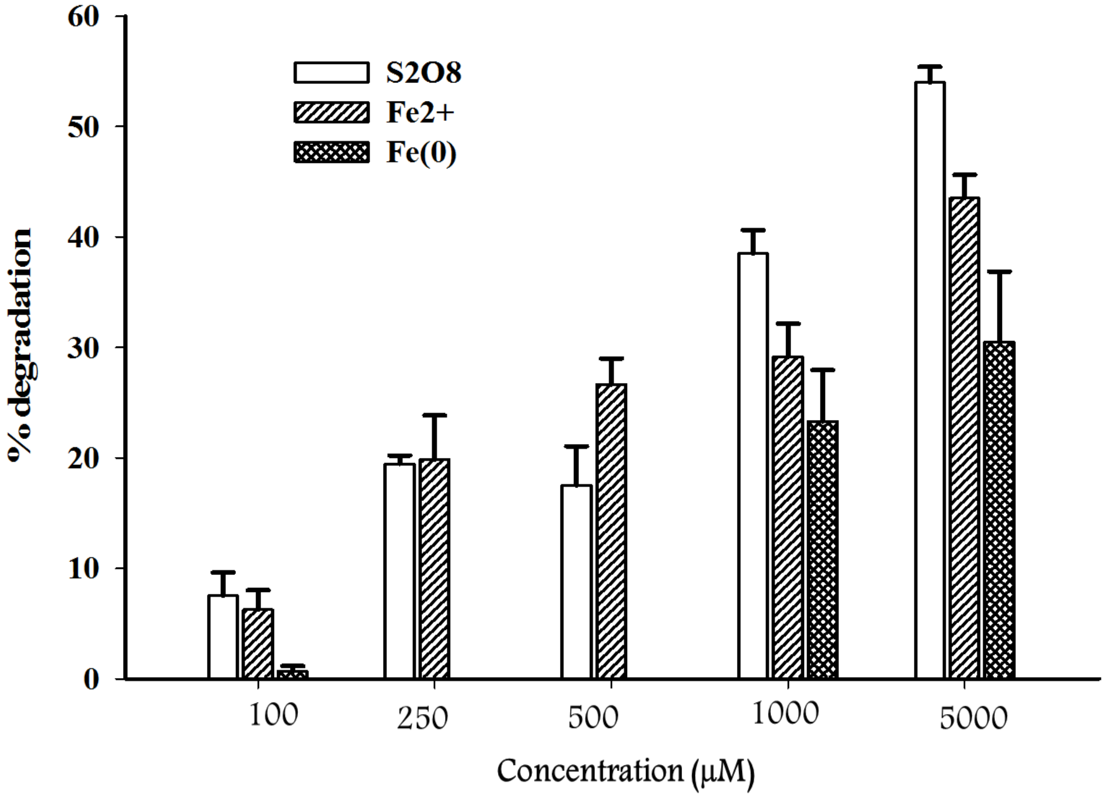


Figure 4.3.2 Degradation of chlortetracycline with persulfate, Fe^{2+} and zero valent iron individually.

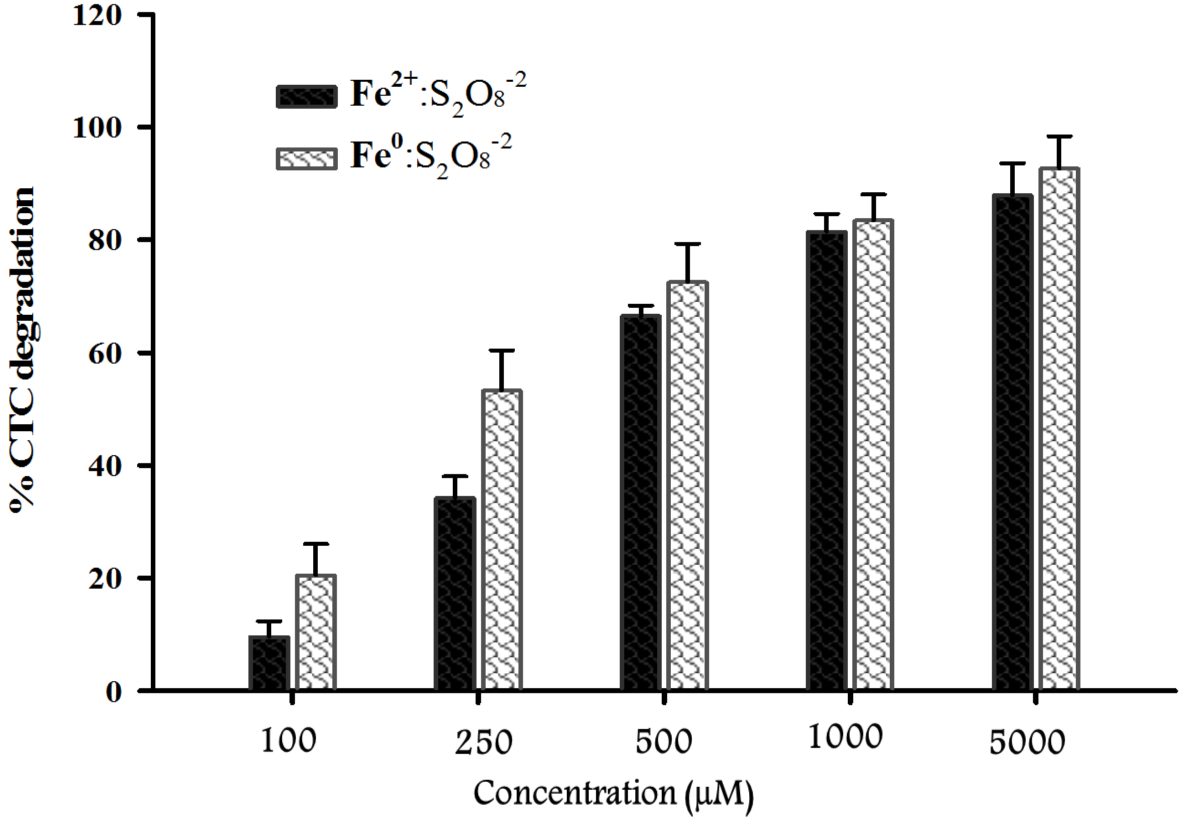


Figure 4.3.3 **Comparison of chlortetracycline (CTC) degradation efficiency of Fe^{2+} and zero-valent iron (Fe^0) by activating persulfate at equal concentration and 1 µM.**

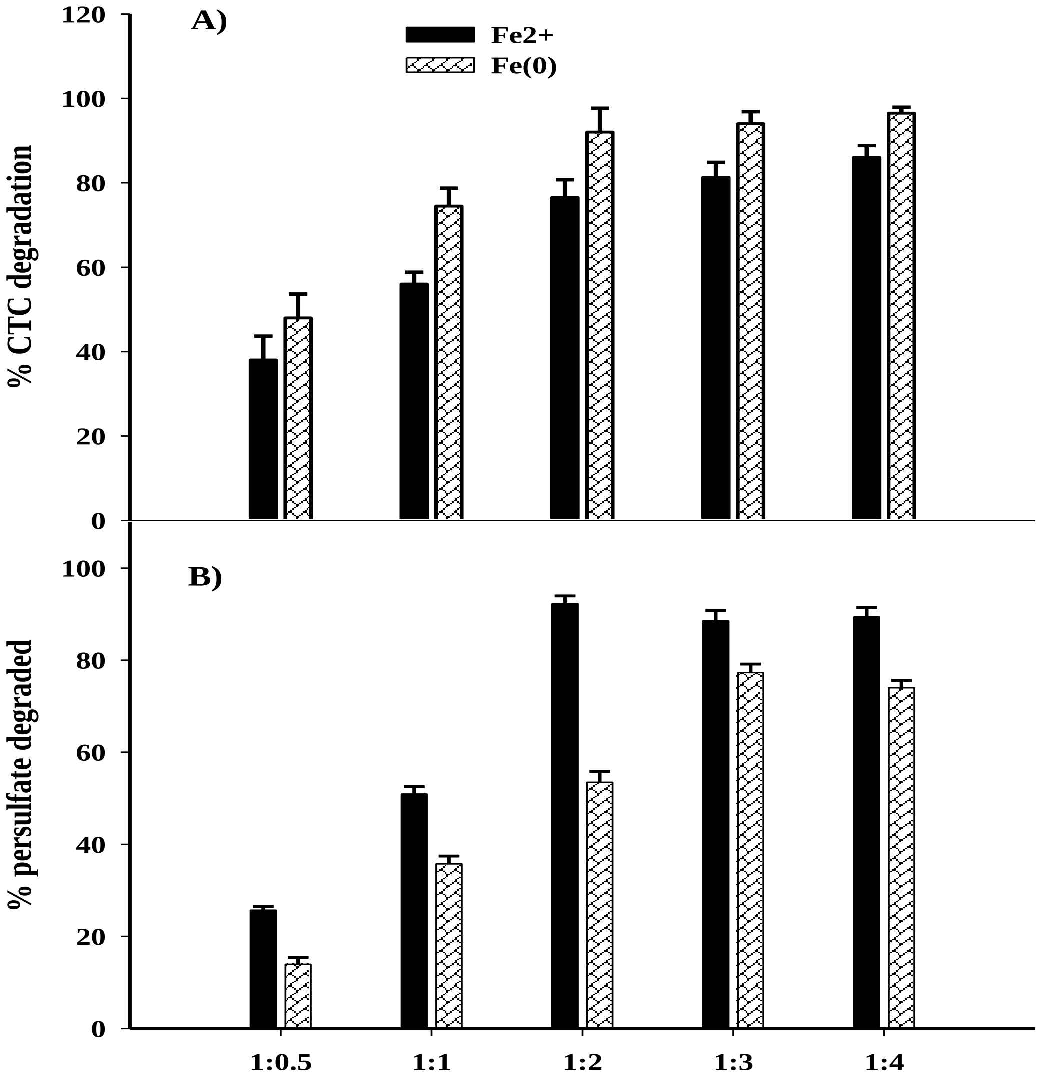


Figure 4.3.4 Optimization of Fe^{2+} and zero valent iron concentration A) degradation of chlortetracycline (CTC) B) reduction of persulfate at 500 μM persulfate and 1 μM CTC

.

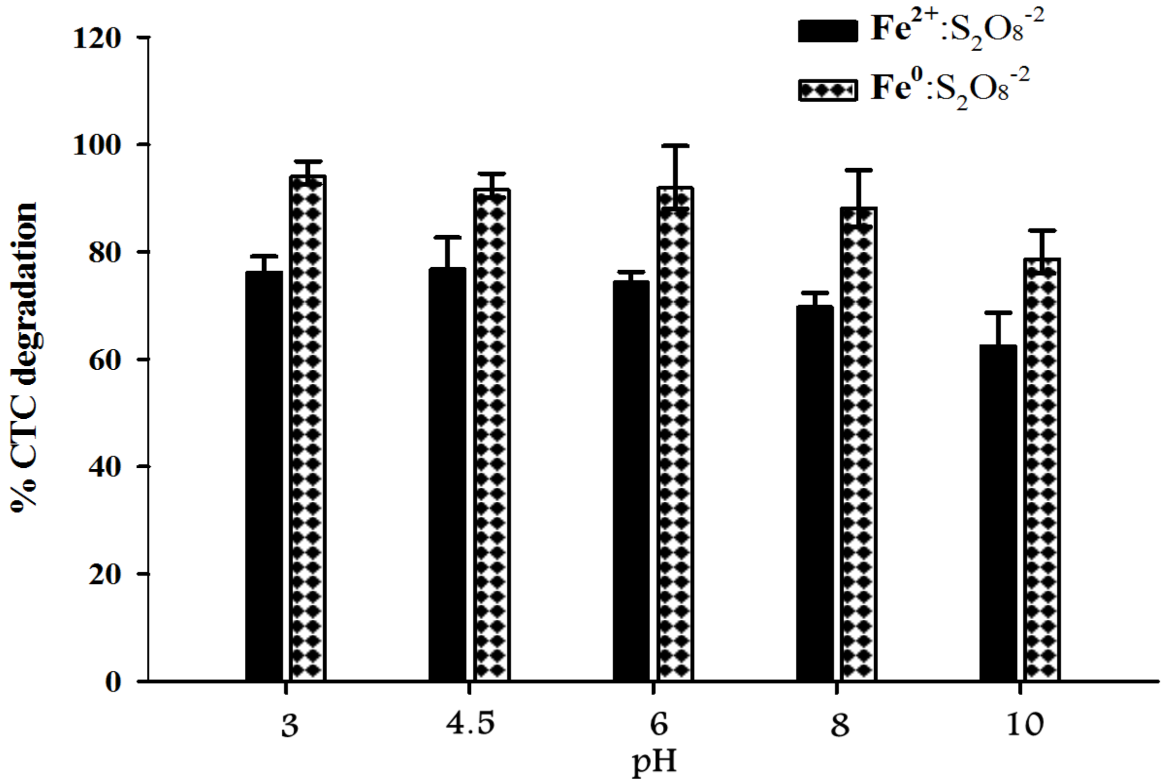


Figure 4.3.5 **The effect of initial pH on the degradation of chlortetracycline (CTC = 1 µM; PS:Fe^{2+}/ZVI; 1:2 = 500:1000 µM).**

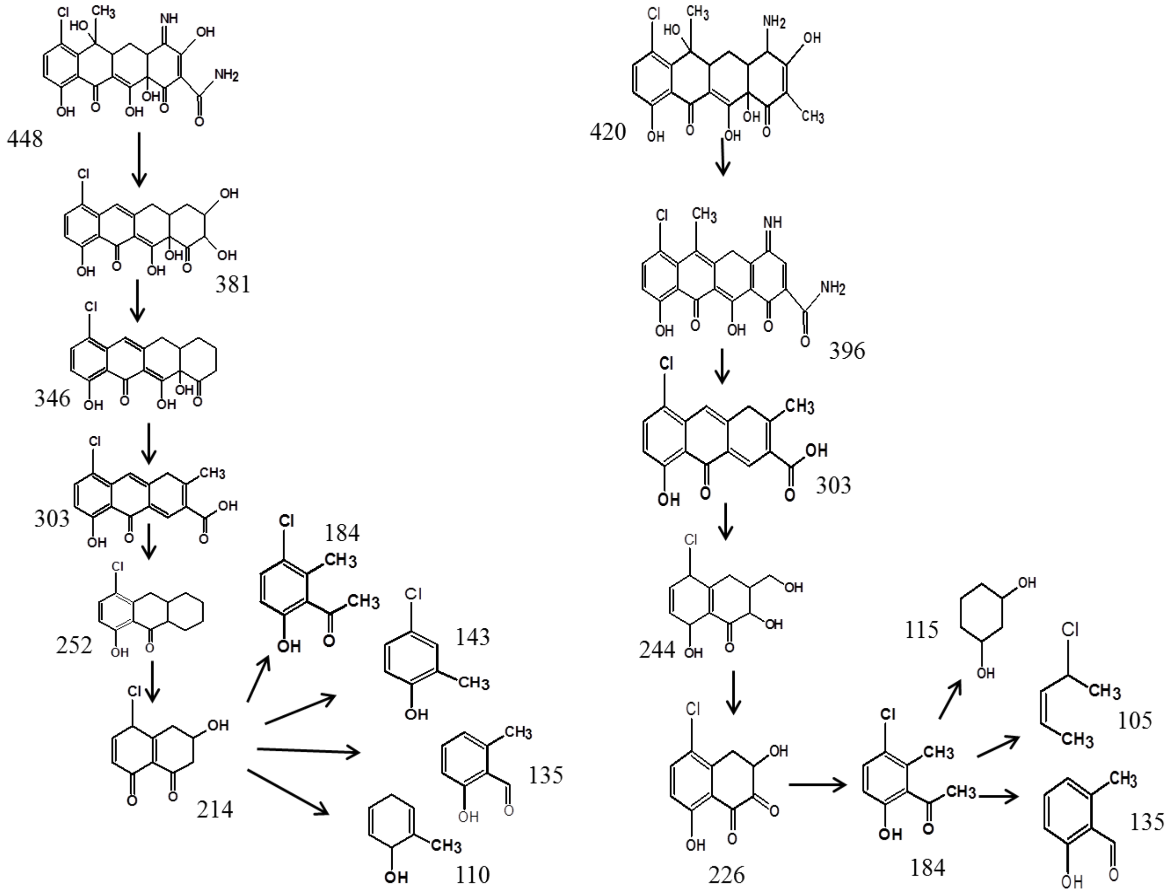


Figure 4.3.6 **Transformation products and proposed degradation pathways of CTC A) PS/Fe^{2+} process B) PS/ZVI process**

CHAPTER 5

COST EVALUATION OF DIFFERENT HYBRID AOPS

PART 1

TECHNO-ECONOMIC ANALYSIS OF HYBRID ADVANCED OXIDATION TREATMENTS IN THE REMOVAL OF CHLORTETRACYCLINE FROM WASTEWATER TREATMENT PLANT

Résumé

Cette étude évalue la faisabilité technico-économique de l'utilisation de procédés d'oxydation avancées hybrides (POA) dans la station de traitement des eaux usées. Les résultats expérimentaux des différents traitements des eaux usées et des boues d'épuration ont été pris en considération pour l'évolution techno-économique de cinq POA différents pour la dégradation de l'antibiotique chlorotétracycline (CTC). L'efficacité de la dégradation a été variée en fonction de la matrice (eaux usées et boues d'épuration) et aussi sur l'application des POA individuels ou hybrides. L'efficacité du traitement a été considérée comme un paramètre technique et le coût comme une limitation économique pour analyser la faisabilité de l'application du processus dans la station d'épuration. Parmi les POA, l'ultrasonisation (UlS), l'oxydation de Fenton (OF) et la ferro-sonication (FS) sont utilisés pour traiter des boues d'épuration, le FS hybride a été considéré comme le meilleur procédé pour traiter les boues d'épuration car ce processus a un coût similaire à celui de la norme unique UlS et plus efficacité puis processus UlS et FO. De même, les procédés de traitement de WW de la casse à ultrasons assistée (UAL) et de la dégradation à base de radicaux sulfate (persulfate / Fe^{2+} / Fe^{0}), UAL pourrait être la meilleure option pour traiter des eaux usées même si l'efficacité de la dégradation de la CTC était inférieure à d'autres processus. Dans le cas des eaux usées, le coût de la dégradation des sulfates était environ 4 fois plus élevé et l'efficacité n'était que de 10 à 14% plus élevée. Par conséquent, tous les paramètres techniques et économiques doivent être pris en compte pour analyser la faisabilité du processus de traitement.

Mots-clés: Technologies d'oxydation avancées ; Voût; Techco-économique; Eaux usée; Boues d'eaux usées.

Abstract

This study evaluates the techno-economic feasibility of using hybrid advanced oxidation technologies (AOTs) in wastewater treatment plant (WWTPs). Experimental results of different wastewater (WW) and wastewater sludge (WWS) treatments were taken into consideration for the techno-economic evolution of five different AOTs for the degradation of antibiotic chlortetracycline (CTC). Degradation efficiency was varied based on the matrix (WW or WWS) and also on the application of individual or hybrid AOPs. The efficiency of treatment was considered as technical parameter and cost as an economical limitation to analyze the feasibility of process application in WWTP. Among AOTs such as ultrasonication (UlS), Fenton oxidation (FO) and ferro-sonication (FS) used to treat WWS, hybrid FS was considered as the best process to treat WWS as this process has a similar cost to standard alone UlS and more efficiency then UlS and FO processes. Similarly, WW treatment processes ultrasonic assisted laccase (UAL) and sulfate radical based degradation (persulfate/Fe^{2+}/Fe^0), UAL might be the best option to treat WW even though the efficiency of CTC degradation was less than other processes. In the case of WW, the cost of sulfate degradation was around 4 times higher and efficiency was only 10-14% higher. Hence, all technical and economical parameters must be taken into consideration to analyze the feasibility of treatment process.

Keywords : Advanced oxidation technologies; Cost; Techco-economic; Wastewater; Wastewater sludge.

Introduction

Current population figures show increased demand for water sources and reuse of treated wastewater (WW) could meet this existing water demand. However, purification of WW that meets the drinking water standards is the main challenge to achieving with currents treatment processes. Further, management of WW is currently an increasingly important global issue due to increasing generation and reuse. The quantity and quality of WW and WWS produced in wastewater treatment plants (WWTPs) are strongly affecting the environmental pollution. Further, on average it was reported that only 60% of WW being treated globally (Mateo-Sagasta *et al.*, 2015), particularly in middle and low-income countries around 8-28% of WW reported being treated (Jiménez *et al.*, 2008). The high cost of WW treatment processes and lack of effective pollution control laws are the main reasons for the existence of few and inefficient WW treatment facilities in most of the developing countries. Since few decades, due to a shortage of fresh water resources reclaimed WW became principal user for agriculture irrigation. According to the studies of Sato et al., 2013, 20-45 million hectares being irrigated with reclaimed WW across the world. This heavy and continuous practice of WW for agriculture can severely damage the environmental and human health.

Most of the WWTPs are using conventional treatments due to the absence of technical and financial sources. However, these methods are efficient to fulfill the current regulations of WW effluent. Nevertheless, generation of by-products like sludge needs further treatment which can be dangerous and not economical to treat. Around 2% of wastewater sludge (WWS) is being produced from the treated WW. Various applications of this generated WWS such as bio-fertilizer, landfilling or biogas production results in magnifying environmental pollution. Further, some types of WW cannot be treated efficiently conventionally due to the presence of high levels of persistent organic compounds. Therefore, the foremost goal in the 21st century is to develop WW treatment technologies that are safe, simple, economical, and the most importantly, it should be efficient in producing WW that is non-harmful to the environment. Advanced oxidation technologies (AOTs) such as UV, ozonation, ultrasonication, Fenton, photocatalysis, and enzymatic treatment have been developed to treat recalcitrant compounds. Many studies proved the efficiency of these technologies in the removal of persistent organic compounds ranging from 30-99%. However, the efficiency of standalone AOTs is changing depending on the environmental conditions and organic compounds. Hence, research has been done in combining AOTs to increase their efficiency and minimizing process limiting factors. Even though studies have approved the increased efficiency of hybrid AOTs but the application in the real WWTPs not yet practiced.

The techno-economical study will decrease the gap in developing and application of these technologies to achieve sustainable development and environmental protection. This study reports the techno-economic assessment of five different WW and WWS treatments from the experimental results conducted in the laboratory to degrade the antibiotic chlortetracycline (CTC). Table 5.1 shows the optimum process details of different AOTs used to treat WW and WWs and their efficiencies. This study aims at providing a detailed comparison of the standalone and hybrid AOTs used in WW and WWS based on the operating cost, plant modeling, and process efficiencies.

Materials and methods

Costs of all stated figures are in Canadian dollars ($Can), which is taken as equivalent to $ 1.33 (dated 10 April 2017) for conversion purposes.

General process details

EXCEL program was used to for the treatment process analysis and economic evaluation of the used AOTs in the degradation of emerging pharmaceutical pollutant CTC in different matrices. Quebec urban community plant having an average daily capacity of generating 204073 m^3/d effluent flow and 54 T/d of sludge was taken into consideration to calculate the cost analysis. Performed standalone and hybrid AOTs efficiencies were compared and the suitable process has been modeled in the existed treatment plant design. The major process parameters include degradation efficiency, the concentration of chemicals, and time of operation.

Wastewater sludge treatment

The general treatment process was not disturbed but the additional treatment step was included at the stage of mixed sludge before dewatering step. Sludge pH was adjusted to 3, and simultaneous addition of H_2O_2 and placing ultrasonic probe was for ferro-sonication (FS) treatment for optimum conditions as mentioned in table 5.1. Similarly, other sludge treatments such as ultrasonication where the only ultrasonic probe was utilized and in the case of Fenton oxidation, Fe^{2+} and H_2O_2 were added (Pulicharla *et al.*, 2017).

Wastewater

After the biological treatment of wastewater, instead of applying ultraviolet treatment; ultrasonic assisted laccase (UAL) degradation was used. In another WW treatment, persulfate was activated using different forms of iron (Fe^{2+} and Fe^0) before UV treatment. Optimum conditions mentioned used for these methods were mentioned in table 5.1. For UAL and Fe^{2+} activated persulfate degradations agitator was installed to solubilize the laccase and Fe^{2+} respectively and the agitation was done only for 30 in 2h of treatment, wherein Fe^0 treatment, agitation was continued for the whole treatment process.

Economic analysis

Studies of (Bertanza *et al.*, 2014, MS Peters, 1980, Max Stone Peters *et al.*, 1968) extensively described the standard protocols to carry out the economic analysis. The WWTP is operated 24h/d and 356 days per year and the capital cast of different equipment were calculated based on the operating parameters of each process. Economic comparison among the treatments was expressed indicating the additional new cost that must be added to the existing WWTP process. Sensitivity analysis was implemented to highlight the cost increasing parameters. The calculations for process installation and equipment cost, total investment cost, operating cost, equipment design were carried out in the EXCEL program. Table 5.2 represents the total capital cost of each process used in this study.

Results and discussion

Analysis of total treatment processes cost

Table 5.2 represents the total treatment cost of all AOTs used for economic analysis in this study. Standard alone AOTs such as UlS and FO processes were approximately needed $ CAD 24K and 80K respectively where combined FO AOT cost was around $ CAD 24K for WWS treatment. For treating WW, UAL process cost was $ CAD 17K where for sulfate radical AOTs cost was between $ CAD 634-641K. Direct process cost which includes raw material, labor, electricity, maintenance, and repair costs and laboratory costs of different AOTs were calculated and are presented in table 5.2. It was found that UAL treatment showed the least raw material and operating costs among all WWS and WW treatment methods where in WWS, UlS showed least operating cost.

Comparison of techno-economic feasibility of different AOTs

The technical feasibility of mentioned AOTs, namely UlS, FO, FS, UAL, PS-Fe0, and PS-Fe2+ were considered, it was found that hybrid FS treatment was efficient in degradation (82%) of CTC from WWS among UlS, FO, and FS processes whereas PS-Fe^0 process showed highest degradation (94%) of CTC among WW treatment processes UAL, PS-Fe^0 and PS-Fe^{2+}. If the economic feasibility was considered, as mentioned previously, UlS and UAL treatments showed the low operating costs among all WWS and WW treatment methods respectively. By taking into the consideration of technical and economical aspects, it was found that FS process for WWS and UAL technique for WW seems to be the best technologies to be applied in the real WWTPs.

Comparing the standard alone AOTs, UlA and FO; FO was costly even though it's resulted in highest efficiency in degradation of CTC. But the combination of these processes FS showed the almost similar cost to UlS and also increased the efficiency of CTC removal. Apart from the raw material cost, remaining cost parameters were similar for both UlS and FS processes as shown in table 5.1. In WW treatment, in contrast to WWS, low-efficiency method UAL was considered as best process due to higher treatment cost of other processes. Sulfate based degradation processes showed around 4 times higher cost compared to UAL, however, the efficiency is only 10-14% more than the UAL degradation. Hence, UAL degradation seems to be the best method to apply in WWTPs. The major cost regulating modules of the in these treatment process were capital cost and overhead cost in case of FO treatment wherein WW, all parameters were affecting the cost in sulfate radical based treatment processes.

Conclusions

Comparing the efficiency of treatments and most of the cost parameters, hybrid FS was the best process for WWS and UAL degradation was best for WW treatment in WWTPs. In the case of WWS treatment, low cost ($ CAD 24K) and higher efficiency (82%) FS was taken as best and low efficiency (80%) and low cost ($ CAD 17K) UAL was taken into consideration for WW. Capital cost and overhead cost were the major cost regulating modules in treatment process in case of FO treatment and all parameters were controlling the cost of sulfate radical based treatment processes. However, the selected AOTs and their cost seem to be showing economic feasibility in applying in the WWTPs.

Acknowledgement

The authors are sincerely thankful to the Natural Sciences and Engineering Research Council of Canada (Discovery Grant 355254 and Strategic Grant), and Ministère des Relations Internationales du Québec (coopération Québec-Catalanya 2012–2014, 01.801-23525) for financial support. Authors are also thankful to “merit scholarship program for foreign students” (FQRNT) for financial assistance to Rama Pulicharla.

References

Bertanza G, Papa M, Canato M, Collivignarelli MC & Pedrazzani R (2014) How can sludge dewatering devices be assessed? Development of a new DSS and its application to real case studies. *Journal of environmental management* 137:86-92.

Jiménez B & Asano T (2008) Water reclamation and reuse around the world. *Water reuse: an international survey of current practice, issues and needs. IWA, London*:3-26.

Mateo-Sagasta J, Raschid-Sally L & Thebo A (2015) Global wastewater and sludge production, treatment and use. *Wastewater,* Springer. p 15-38.

Peters M (1980) Timmerhaus. *KD Plant Design and Economics for Chemical Engineers. McGraw-Hffl, New York. NY*:635-638.

Peters MS, Timmerhaus KD, West RE, Timmerhaus K & West R (1968) *Plant design and economics for chemical engineers.* McGraw-Hill New York,

Pulicharla R, Brar SK, Rouissi T, Auger S, Drogui P, Verma M & Surampalli RY (2017) Degradation of chlortetracycline in wastewater sludge by ultrasonication, Fenton oxidation, and ferro-sonication. *Ultrasonics Sonochemistry* 34:332-342.

Table 5.1 Optimum process parameters of different AOTs and their chlortetracycline degradation efficiencies in different matrices.

Matrix of treatment	AOT	Optimum process details	Efficiency
Wastewater sludge	**Ultrasonication**	Ultrasonic treatment for 106 min at 60% amplitude	**67%**
	Fenton oxidation	Fenton oxidation at 1:1 H_2O_2: Fe^{2+} ratio for 106 min	**76%**
	Ferro-sonication	Ferro-sonication for 106 min at 60% amplitude and H_2O_2 concentrations is equal to fenton oxidation	**82%**
Wastewater	**Ultrasonic assisted laccase degradation**	Bath sonication with laccase of 0.5 IU at 4.5 pH for 2h	**80%**
	Sulfate radical degradation	Fe^{2+} activation of persulfate (PS) for 2h at 1:2, PS:iron)	**92%**
		Zero valent iron activation of persulfate for 2h at 1:2, PS:iron)	**94%**

Table 5.2 **Cost analysis of different advanced oxidation processes used in treating wastewater and wastewater sludge**

Treatment process		Raw material cost ($Cad/day)	Capital cost ($Cad)	Direct operating cost ($Cad/day)	Indirect operating cost and overhead ($Cad/day)	Total treatment cost (CC+operating cost/day) ($Cad)
Ultrasonication		-	20500	1686.453	2016.17	24214.07
Fenton oxidation		925.44	762602.1	16847.43	23049.23	802498.7
Ferro-sonication		186.98	20500	1884.89	2016.17	24401.05
Ultrasonic assisted laccase		27.14	14350	1309.67	1442.7	17102.37
Sulfate radical degradation	Fe0-persulfate	31124.22	574200.9	43047.02	17377.23	634625.1
	Fe2+-persulfate	37967.99	574200.9	49997.55	17377.23	641575.7

CHAPTER 6

CONCLUSIONS AND RECOMMANDATIONS

CONCLUSIONS

Les conclusions suivantes peuvent être tirées du travail accompli:

1) Cette étude a démontré que la concentration de CTC dans les effluents et dans les boues étaient respectivement de 7,28 µg/L et de 0,4-0,8 mg/kg. Ces concentrations détectées de CTC dans la station d'épuration du Québec remettent en cause la libération d'effluents dans l'environnement, la réutilisation et le recyclage des BE (Boues d’Epuration).

2) Une concentration élevée de CTC est présente dans les BE même si elle est soluble dans l'eau (8,6 mg / mL). La forte chélation et la grande capacité d'adsorption de la CTC affectent la distribution de celle-ci. L’estimation des concentrations de métaux dans les EU et les BE, et la corrélation de cette concentration élevée en métal avec une concentration élevée en CTC dans la matrice correspondante, supportent la rétention de la CTC dans les BE.

3) L'analyse de CTC en utilisant LDTD-MS / MS a réduit le temps d'analyse de quelques minutes (20 min) à quelques secondes (10 s) et il est sans solvant pendant l'analyse d'échantillon. La méthode développée pour l'analyse de la CTC à l'aide de LDTD-MS / MS était une méthode d'analyse nouvelle, plus rapide et sans solvant.

4) Les constantes de stabilité des complexes CTC-métal suivent l'ordre: Mg-CTC> Ca-CTC> Cu-CTC> Cr-CTC. Ceci explique la forme chimique stable de la CTC dans la STEP.

5) En raison des différences caractéristiques inhérentes dans la composition de la paroi cellulaire des bactéries Gram-positives et Gram-négatives, elles ont montré une différence dans l'accessibilité au CTC et à ses complexes métalliques. Les complexes CTC-métal ont présenté une toxicité envers des Bt dans l'ordre suivant: Mg-CTC> Cu-CTC> Ca-CTC ≡ Cr-CTC. La toxicité la plus élevée du complexe Mg-CTC dans le Bt était due à une affinité de liaison plus élevée du CTC avec le Mg (II) et aussi un rôle

biologique important des ions de Mg. Les complexes CTC-métal ont montré une différence de toxicité négligeable (p> 0,05) pour Ea.

6) Une plus faible toxicité de CTC a été observée dans les échantillons réels de BE en raison de l'adsorption plus élevée de CTC dans la partie solide de boues qui n'est biologiquement pas disponible pour les microorganismes.

7) Les processus de nitrification et de dénitrification de la station d'épuration ont été inhibés à des concentrations plus élevées de CTC. Dans la nitrification, l'élimination de la DCO et la cinétique d'oxydation de l'ammoniac ont été inhibées jusqu'à 50% à 10 mg de CTC / L. De même, la cinétique d'oxydation des nitrites a été inhibée de 50% à 0,5 mg de CTC / L. Pendant la dénitrification, 14 et 10 mg / L de CTC ont inhibé l'élimination du DCO et la cinétique d'oxydation des nitrites, respectivement.

8) FS a montré 82%, tandis que les procédés de FO et de UlS ont montré 67% et 76% de dégradation de la CTC, respectivement. La libération de CTC par ultrasonication et l’utilisation de fer à pH 3 (FO) ont été les principaux facteurs montrant un effet synergique sur la dégradation de la CTC dans le traitement FS.

9) La dégradation de la CTC par des radicaux sulfate dans les procédés PS / Fe2 + et PS / ZVI a montré 76% et 94% respectivement à un rapport molaire 1: 2 pour 1 mM de CTC en 2h à 500 µM de PS et 1000 µM de fer. Comparée à la production plus élevée de radicaux sulfate à la fois (PS / Fe2 +) avec le catalyseur homogène, la production lente dans le processus hétérogène (PS / ZVI) semble être efficace.

10) La CTC a été éliminée à plus de 80% en 2h dans le traitement par UAL, alors que la laccase utilisée seule a mis 2 jours pour dégrader 87% de CTC. Comparée à la laccase seule, la technique d’UAL a non seulement augmenté la dégradation de la CTC (~ 80%) mais a également réduit le temps de dégradation (2h).

11) Les résultats de l'analyse de l'Estrogen Screen de levure ont montré que les échantillons traités avec UlS, FO, FS, PS et UAL n'avaient pas présenté d'activité oestrogénique.

RECOMMANDATIONS POUR L'AVENIR

À partir des résultats obtenus sur l'étude du devenir et des différentes méthodes de dégradation de la CTC dans la station d'épuration, les recommandations suivantes peuvent être envisagées:

1) Les propriétés de complexation et d'adsorption de toutes les classes de contaminants émergents doivent faire l'objet d'une étude pour déterminer le partitionnement complet dans la station d'épuration.

2) Comme les antibiotiques diminuent l'efficacité des processus biologiques de la station d'épuration, et les bactéries à leur tour développent une résistance sous la pression sélective en raison de l'exposition à des antibiotiques de faible niveau, il est préférable de développer des moyens pour traiter les eaux usées avant d'initier un traitement biologique pour diminuer le développement de la résistance et augmenter l'efficacité des processus biologiques.

3) Une plus grande détection des antibiotiques dans les boues exige une recherche plus approfondie pour le traitement des eaux usées. La majeure partie de la littérature est axée sur la dégradation des contaminants traces dans l'eau potable, les effluents des eaux de surface et des eaux usées. Très peu d'études ont été faites sur les boues d'épuration, qui est le puits principal. Par conséquent, le développement des processus avancés pour les boues d'épuration dans la station d'épuration des eaux usées est nécessaire et son efficacité doit être surveillée.

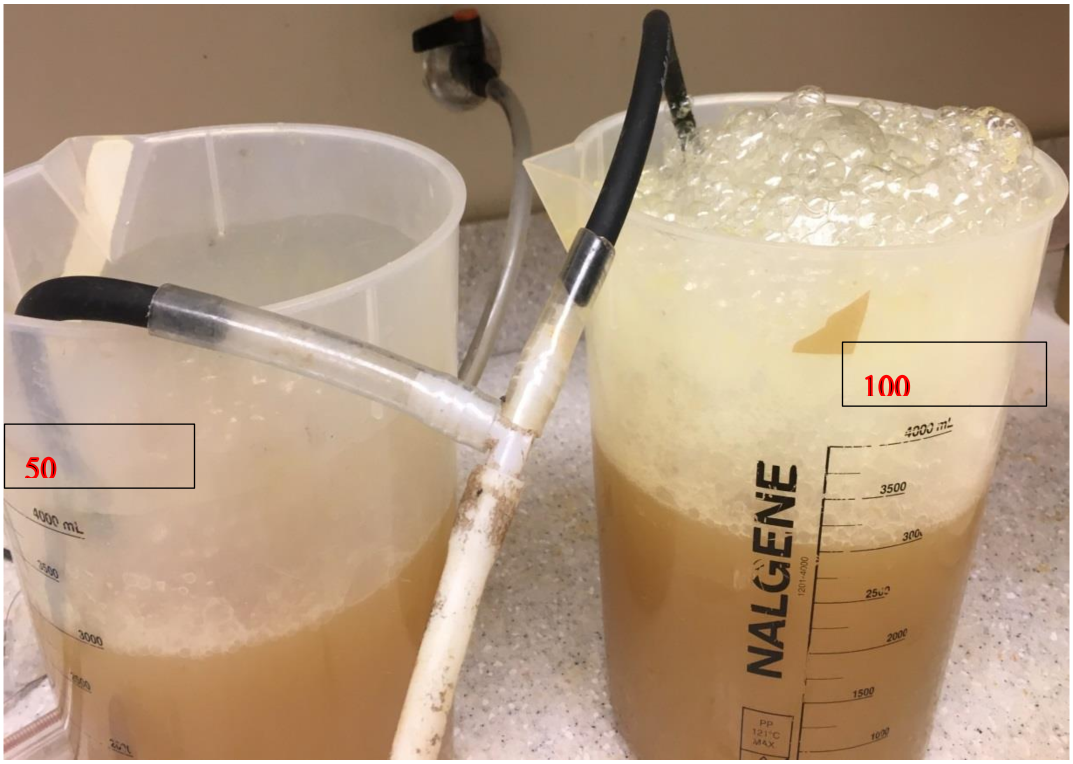
4) Plus d'investigations sont nécessaires, en particulier sur l'application combinée/hybride des POA pour accroître leur efficacité par leurs effets synergiques et les rendre économiquement réalisables et respectueux pour l'environnement.

ANNEXES

ANNEXES I

CHAPTER 3 PART 2

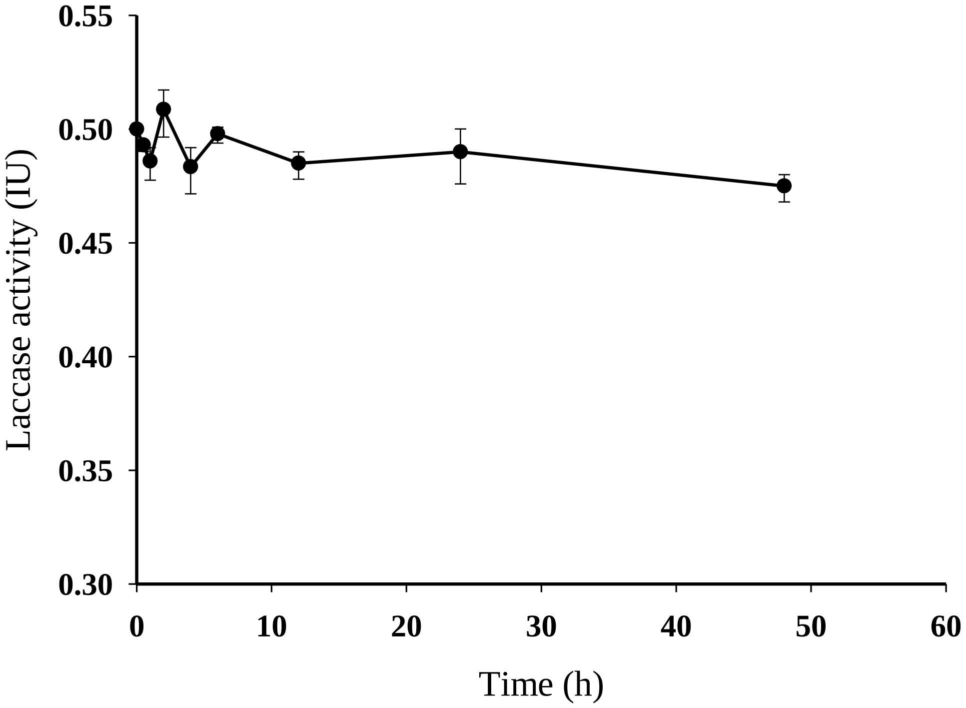
DATA: Bubble formation in aerobic reactors of 50 and 100 mgCTC/L experiments



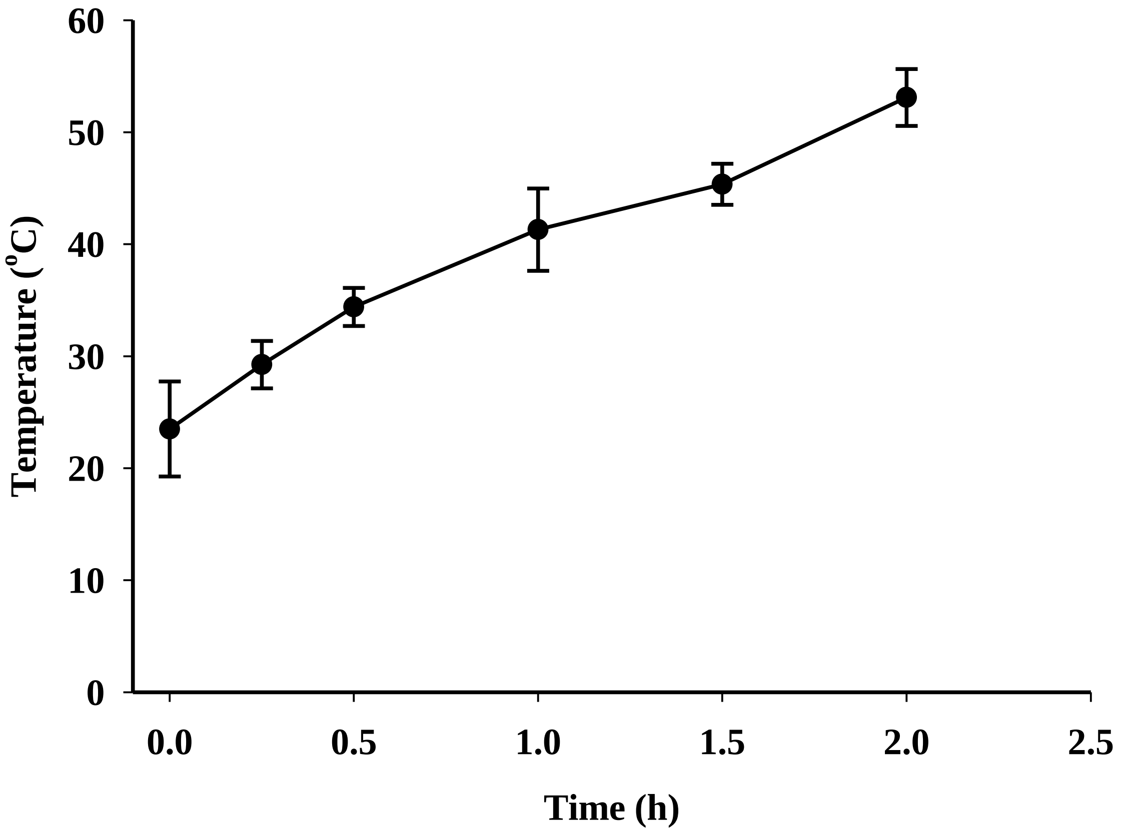
ANNEXES II

CHAPTER 4 PART 2

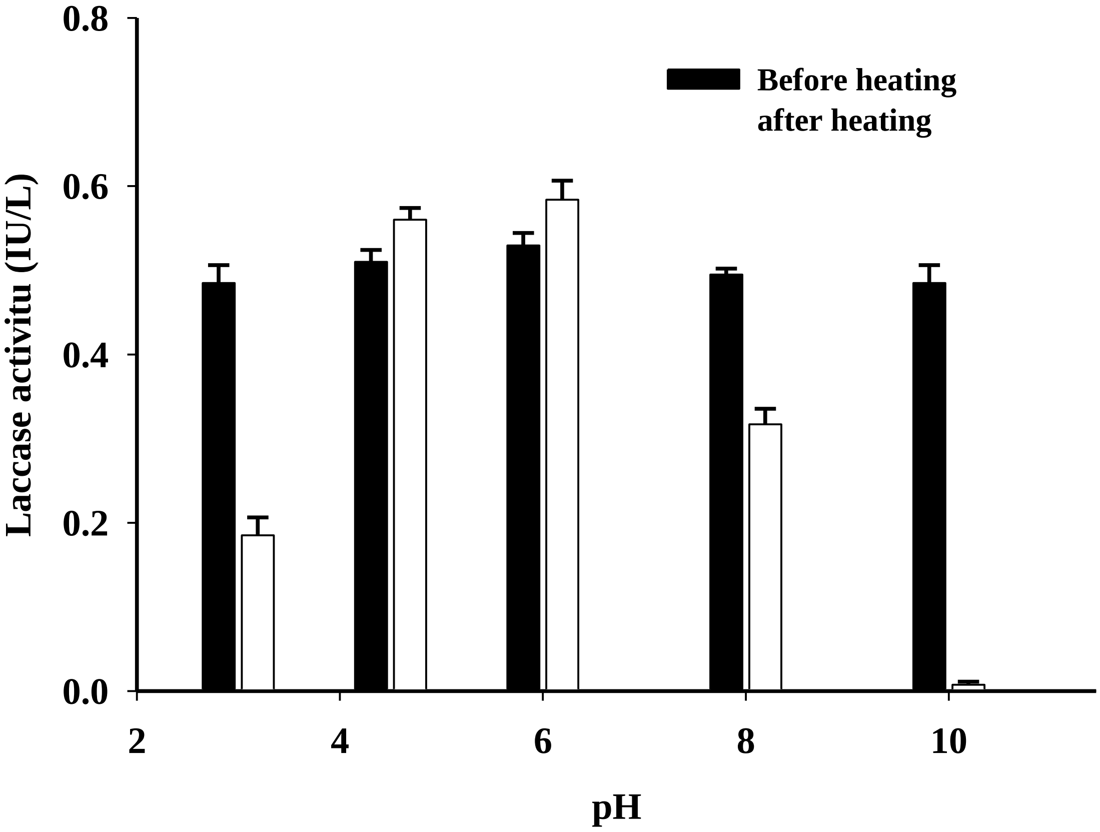
DATA 1: **Stability kinetics of laccase during the degradation of chlortetracycline in wastewater for 48h at room temperature**



DATA 2: Changes in temperature of wastewater during ultrasonic assisted degradation of chlortetracycline



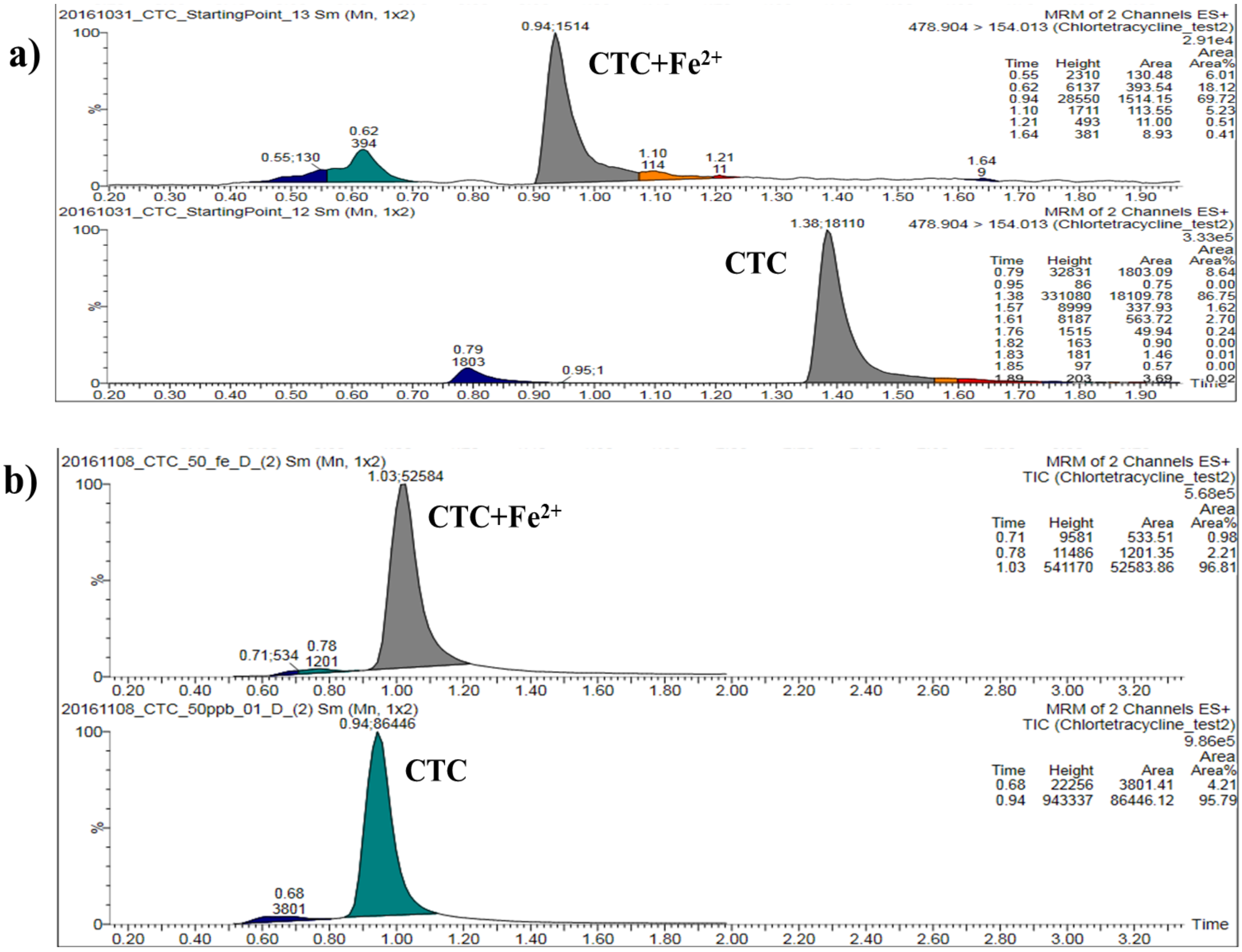
DATA 3: Effect of temperature on laccase activity in wastewater



ANNEXES II

CHAPTER 4 PART 3

DATA: CTC elution peaks in UHPLC-MS/MS before and after complexation with iron. a) After 30 min complexation; b) after 12 h complexation



Time	Height	Area	Area Area%
0.55	2310	130.48	6.01
0.62	6137	393.54	18.12
0.94	28550	1514.15	69.72
1.10	1711	113.55	5.23
1.21	493	11.00	0.51
1.64	381	8.93	0.41

Time	Height	Area	Area Area%
0.79	32831	1803.09	8.64
0.95	86	0.75	0.00
1.38	331080	18109.78	86.75
1.57	8999	337.93	1.62
1.61	8187	563.72	2.70
1.76	1515	49.94	0.24
1.82	163	0.90	0.00
1.83	181	1.46	0.01
1.85	97	0.57	0.00
1.89	203	3.69	0.02

Time	Height	Area	Area Area%
0.71	9581	533.51	0.98
0.78	11486	1201.35	2.21
1.03	541170	52583.86	96.81

Time	Height	Area	Area Area%
0.68	22256	3801.41	4.21
0.94	943337	86446.12	95.79