University of the Aegean

Department of Environment

PhD dissertation

Investigation on the Fate of Benzotriazoles and Benzothiazoles during Biological Wastewater Treatment

Mazioti Aikaterini-Anna
Environmental Engineer

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Μαζιώτη Αικατερίνη-Άννα
Μηχανικός Περιβάλλοντος

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Thesis Advisory Committee

Athanasios Stasinakis, Assistant Professor, Department of Environment, University of the Aegean (supervisor)

Nikolaos Thomaidis, Associate Professor, Department of Chemistry, National and Kapodistrian University of Athens

Daniel Mamais, Associate Professor, School of Civil Engineering, National Technical University of Athens

Thesis Examination Committee

Athanasios Stasinakis, Assistant Professor, Department of Environment, University of the Aegean (supervisor)

Nikolaos Thomaidis, Associate Professor, Department of Chemistry, National and Kapodistrian University of Athens

Daniel Mamais, Associate Professor, School of Civil Engineering, National Technical University of Athens

Pilinis Christodoulos, Professor, Department of Environment, University of the Aegean

Evan Diamadopoulos, Professor, School of Environmental Engineering, Technical University of Crete

Danae Venieri, Assistant Professor, School of Environmental Engineering, Technical University of Crete

Nikolaos Xekoukoulotakis, Assistant Professor, School of Environmental Engineering, Technical University of Crete

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Νικόλαος Θωμαΐδης, Αναπληρωτής Καθηγητής, Τμήμα Χημείας, Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών

Δανιήλ Μαμάης, Αναπληρωτής Καθηγητής, Σχολή Πολιτικών Μηχανικών, Εθνικό Μετσόβιο Πολυτεχνείο

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ΤΜΗΜΑ ΠΕΡΙΒΑΛΛΟΝΤΟΣ

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6. Νικόλαος Ξεκουκουλωτάκης, Επίκουρος Καθηγητής, Σχολή Μηχανικών Περιβάλλοντος, Πολυτεχνείο Κρήτης
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«Investigation on the Fate of Benzotriazoles and Benzothiazoles during Biological Wastewater Treatment»

(Διερεύνηση της Τύχης Βενζοτριαζολών και Βενζοθειαζολών κατά τη Βιολογική Επεξεργασία Υγρών Αποβλήτων)

Η εξεταστική επιτροπή αξιολόγησε την διδακτορική διατριβή της κας **Αικατερίνης-Άννας Μαζιώτη** και ...ομόφωνα... αποφασίζει ότι:

- Το περιεχόμενο της διατριβής είναι πρωτότυπο
- Συμβάλει ουσιαστικά στην επιστήμη
- Η παρουσίαση από τον υποψήφιο είναι .Άριστη

Με βάση τα παραπάνω η επταμελής επιτροπή προτείνει την απονομή διπλώματος **της Διδάκτορα των Επιστημών του Περιβάλλοντος** στην κα **Αικατερίνη΄-Άννα Μαζιώτη** με βαθμό .Άριστα, Δέκα (10)

Η Επιτροπή

Στασινάκης Αθανάσιος	**Μαμάης Δανιήλ**	**Θωμαΐδης Νικόλαος**	**Πηλίνης Χριστόδουλος**	**Διαμαντόπουλος Ευάγγελος**	**Ξεκουκουλωτάκης Νικόλαος**	**Βενιέρη Δανάη**
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Abstract

A major problem concerning wastewater treatment nowadays is the elimination of organic micropollutants from raw municipal and industrial wastewater. Many groups of compounds, such as surfactants, personal care products, pharmaceuticals, estrogens, perfluorinated compounds, phthalate acid esters and others are thoroughly examined concerning their occurrence and removal from wastewater as well as their ecotoxicity to living organisms. During this study benzotriazoles (BTRs) and benzothiazoles (BTHs) were examined regarding their biological removal from sewage. BTRs and BTHs are used in many industrial and every day products, leading to their presence in wastewater. Their frequent detection in surface water indicates their inadequate elimination during wastewater treatment. So far, little is known about the biodegradation rates of BTRs and BTHs by suspended and attached biomass and about their removal efficiencies in different biological wastewater treatment systems. The main goals of this study was a) to investigate the fate of BTRs and BTHs during biological wastewater treatment, as well as the role of biodegradation and sorption on their removal and b) to compare BTRs and BTHs removal efficiency in different biological treatment systems (activated sludge system, AS; moving bed biofilm reactor system, MBBR; hybrid moving bed biofilm reactor system, HMBBR). More specifically, 1H-benzotriazole (BTR), 5-chlorobenzotriazole (CBTR), xylytriazole (XTR), 4-methyl-1H-benzotriazole (4TTR), 5-methy-1H-lbenzotriazole (5TTR) and 2-hydroxy-benzothiazole (OHBTH) were studied and experiments were conducted in three steps.

In the first step, BTRs and BTHs sorption and biodegradation onto activated sludge (AS) was investigated in batch experiments. Experiments with sterilized AS showed no abiotic transformation of these compounds, while their sorption constants ranged between 87 (XTR) and 220 L Kg^{-1} (BTR). Regarding the biodegradation experiments, the influence of different conditions was examined as to the target compounds treatment with AS. The presence of easily degradable organic compounds enhanced their biodegradation, showing that these compounds are mainly removed as a result of co-metabolism. The half lives calculated in batch experiments varied between 6.5 h for OHBTH to 47 h for CBTR. The different SRT of AS did not seem to influence biodegradation of target compounds. Concerning the fate of target compounds in full-scale STPs, the application of appropriate equations showed that the examined compounds are expected to be partially removed mostly through aerobic biodegradation, while sorption poorly contributes to their elimination from sewage (less than 3%).

In the second experimental part, the biodegradation of BTRs and BTHs in lab-scale AS and MBBR systems was studied. Both systems were able to remove target compounds at different rates. Removal efficiencies ranged from 43% to 76% for BTR, 8% to 69% for 4TTR, 0% to 53% for 5TTR, 42% to 49% for CBTR, 9% to 43% for XTR and 80% to 97% for OHBTH. The attached biomass (MBBR) presented higher biodegradation constants (k_{bio}, L g_{SS}^{-1} d^{-1}) compared to suspended biomass (AS). The operational parameters of each system seemed to strongly influence the microbial community that was developed, leading to fluctuation in removal in each system. The biomass developed in the MBBR system presented higher specific removal rates of the target compounds. In general, specific removal rates in the MBBR system reached 11.9 (BTR), 15.1 (4TTR), 14.4 (5TTR), 11.3 (CBTR), 9.7 (XTR) and 13.6 (OHBTH) μg of micropollutant removed per g of biomass per day. Two experimental cycles were conducted for the MBBR system, testing the influence of organic loading on the removal capacity of the system. According to the results, higher micropollutants removal rates were obtained when the MBBR system was operated under low organic loading conditions.

In the last experimental part of this PhD Thesis, a HMBBR system was used and the removal efficiency of target compounds was investigated. According to the results, the total removal rates obtained were 75% (BTR), 41% (4TTR), 57% (5TTR), 61% (CBTR), 74% (XTR) and 81% (OHBTH). Biodegradation of target compounds occurred mainly in the first reactor of the HMBBR, while the second reactor contributed significantly to the removal of the most resistant compounds (4TTR). The contribution of each type of biomass that co-exists in a HMBBR systems was examined, by using biodegradation constants calculated for each type of biomass in batch experiments. For three compounds (OHBTH, BTR and XTR), the main removal mechanism was biodegradation by AS in the first bioreactor. For CBTR and 5TTR, biodegradation by AS and biofilm was almost equal in both bioreactors, while 4TTR was mainly removed by the biofilm developed in the second bioreactor. Possible by-products were investigated with batch biodegradation experiments. In total, twenty-two transformation products were tentatively identified; hydroxylation, oxidation and methylation were the main reaction mechanisms. When compared to systems examined in the second experimental part, the HMBBR performance was similar to a low loaded pure MBBR system and more efficient than AS and MBBR systems operating under the same HRT and organic loading conditions.

The following chapters structure this dissertation: Chapter 1 includes a short literature review on the main wastewater treatment processes used in this study and the target micropollutants investigated, as

well as the objectives and the outline of this PhD Thesis. In Chapter 2, the experimental procedures and analytical methods are described. In Chapter 3, the results of this study are presented and discussed, while Chapter 4 summarizes the most important conclusions as well as suggestions for future research. Thereupon, supplementary data is presented as well as the three publications in scientific journals that came out of this study.

Keywords

benzotriazoles (BTRs), benzothiazoles (BTHs), biological treatment, sewage, biotransformation, sorption, activated sludge, moving bed biofilm reactor, biofilm, biocarriers

Περίληψη

Ένα σημαντικό πρόβλημα όσον αφορά στην επεξεργασία των υγρών αποβλήτων είναι η απομάκρυνση οργανικών μικρορύπων από αστικά και βιομηχανικά λύματα. Πολλές ουσίες, όπως τασιενεργές ουσίες, προϊόντα προσωπική περιποίησης, φαρμακευτικές ουσίες, οιστρογόνα, υπερφθωριωμένες ενώσεις, φθαλικοί εστέρες και άλλες, έχουν μελετηθεί όσον αφορά στην εμφάνιση και απομάκρυνση από τα λύματα αλλά και όσον αφορά στην τοξικότητά τους σε ζωντανούς οργανισμούς. Κατά την διεξαγωγή της παρούσας μελέτης, τα βενζοτριαζόλια (BTRs) και τα βενζοθειαζόλια (BTHs) εξετάστηκαν όσον αφορά στη βιολογική απομάκρυνσή τους από τα λύματα. Τα βενζοτριαζόλια και τα βενζοθειαζόλια χρησιμοποιούνται ευρέως σε βιομηχανικές εφαρμογές και σε προϊόντα καθημερινής χρήσης, προκαλώντας την παρουσία τους στα υγρά απόβλητα. Η ανίχνευσή τους στα επιφανειακά ύδατα μας προϊδεάζει για την ανεπαρκή απομάκρυνσή τους κατά την επεξεργασία των υγρών αποβλήτων. Λίγες είναι οι πληροφορίες σχετικά με τους ρυθμούς βιοαποδόμησης αυτών των ουσιών από βιομάζες διαφορετικού τύπου καθώς και για τα ποσοστά απομάκρυνσής τους σε διάφορα συστήματα βιολογικής επεξεργασίας αποβλήτων. Οι κύριοι στόχοι της παρούσας εργασίας ήταν α) η διερεύνηση της τύχης βεζοτριαζολίων και βενζοθειαζολίων κατά τη βιολογική επεξεργασία υγρών αποβλήτων, καθώς και ο ρόλος της βιοαποδόμησης και της προσρόφησης στην απομάκρυνσή τους και β) η σύγκριση της απομάκρυσής τους σε διαφορετικά συστήματα βιολογικής επεξεργασίας (ενεργού ιλύος, αντιδραστήρων κινούμενης κλίνης με βιοφορείς, υβριδικό σύστημα). Πιο συγκεκριμένα, οι ουσίες βενζοτριαζόλη (BTR), χλωρο-βενζοτριαζόλη (CBTR), ξυλιτριαζόλη (XTR), 4-μέθυλο-βενζοτριαζόλη (4TTR), 5-μέθυλο-βενζοτριαζόλη (5TTR) και η ύδροξυ-βενζοθειαζόλη (OHBTH) εξετάστηκαν και πραγματοποιήθηκαν πειράματα σε τρία στάδια.

Σε πρώτη φάση, η προσρόφηση και η βιοαποδόμηση των υπό μελέτη ουσιών εξετάστηκαν στην ενεργό ιλύ μέσω πειραμάτων διαλείποντος έργου. Πειράματα με αδρανοποιημένη ενεργό ιλύ έδειξαν οτι οι ουσίες δεν διασπώνται αβιοτικά, ενώ οι σταθερές προσρόφησής των ουσιών κυμάνθηκαν από 80 (XTR) έως 220 L Kg^{-1} (BTR). Σχετικά με τα πειράματα βιοαποδόμησης, η επίδραση ορισμένων παραμέτρων εξετάστηκε για την απομάκρυνση των ουσιών με ενεργό ιλύ. Η παρουσία εύκολα διασπάσιμων οργανικών ενώσεων επιτάχυνε την βιοδιάσπασή τους, υποδεικνύοντας ότι οι συγκεκριμένες ουσίες απομακρύνονται μέσω συμμεταβολισμού. Οι χρόνοι ημιζωής υπολογίστηκαν με πειράματα διαλείποντος έργου και κυμάνθηκαν από 6.5 h για την

OHBTH μέχρι 47 h για την CBTR. Διαφορετικοί χρόνοι παραμονής των στερεών (SRT) στην ενεργό ιλύ δεν φάνηκε να επηρεάζουν τη βιοαποδόμηση των ουσιών. Σχετικά με την τύχη των ουσιών σε συστήματα επεξεργασίας μεγάλης κλίμακας, η εφαρμογή των κατάλληλων εξισώσεων έδειξε ότι οι υπό μελέτη ουσίες αναμένεται να απομακρύνονται μερικώς μέσω της δεξαμενής αερισμού, ενώ η απομάκρυνσή τους μέσω της προσρόφησή τους στα στερεά δεν ήταν σημαντική (μικρότερη από 3%).

Στην δεύτερη πειραματική φάση εξετάστηκε η βιοαποδόμηση βενζοτριαζολίων και βενζοθειαζολίων σε σύστημα ενεργού ιλύος και σε σύστημα αντιδραστήρων κινούμενης κλίνης με βιοφορείς. Και τα δυο συστήματα αποδείχτηκαν ικανά να απομακρύνουν τις ουσίες σε διαφορετικό βαθμό. Τα ποσοστά απομάκρυνσης κυμάνθηκαν από 43% μέχρι 76% για την BTR, 8% μέχρι 69% για την 4TTR, 0% μέχρι 53% για την 5TTR, 42% μέχρι 49% για την CBTR, 9% μέχρι 43% για την XTR και 80% μέχρι 97% για την OHBTH. Η προσκολλημένη βιομάζα στο δεύτερο σύστημα παρουσίασε υψηλότερες σταθερές βιοαποδόμησης (kbio, L gSS-1 d-1) σε σχέση με την ενεργό ιλύ. Οι παράμετροι λειτουργίας του κάθε συστήματος επηρέασαν σημαντικά τη μικροβιακή κοινότητα που αναπτύχθηκε σε κάθε περίπτωση, οδηγώντας στις διαφοροποιήσεις που παρατηρήθηκαν στην απομάκρυνση. Η προσκολλημένη βιομάζα παρουσίασε μεγαλύτερη ειδική απομάκρυνση των ουσιών. Οι τιμές ειδικής απομάκρυνσης στο MBBR σύστημα έφτασαν τα 11.9 (BTR), 15.1 (4TTR), 14.4 (5TTR), 11.3 (CBTR), 9.7 (XTR) and 13.6 (OHBTH) μg ουσίας που απομακρύνθηκαν ημερησίως ανα g βιομάζας. Δυο πειραματικοί κύκλοι πραγματοποιήθηκαν για το σύστημα αντιδραστήρων κινούμενης κλίνης με βιοφορείς, ώστε να ερευνηθεί η επίδραση της οργανικής φόρτισης στην απόδοση του συστήματος. Σύμφωνα με τα αποτελέσματα, υψηλότερα ποσοστά απομάκρυνσης επιτεύχθηκαν όταν το σύστημα λειτούργησε σε συνθήκες χαμηλής φόρτισης.

Στο τελευταίο πειραματικό στάδιο ένα υβριδικό σύστημα εξετάστηκε για την απομάκρυνση των ουσιών, το οποίο συνδύαζε τις δυο τεχνολογίες που εξετάστηκαν πρωτύτερα (ενεργού ιλύος και αντιδραστήρων κινούμενης κλίνης με βιοφορείς). Σύμφωνα με τα αποτελέσματα, οι συνολικές απομακρύνσεις που παρατηρήθηκαν ήταν 75% (BTR), 41% (4TTR), 57% (5TTR), 61% (CBTR), 74% (XTR) και 81% (OHBTH).. Η βιοαποδόμηση των ουσιών πραγματοποιήθηκε κυρίως στον πρώτο αντιδραστήρα του συστήματος, ενώ ο δεύτερος αντιδραστήρας συνέβαλε στην απομάκρυνση της 4TTR. Η συνεισφορά κάθε τύπου βιομάζας (βιοφιλμ, ενεργός ιλύς) που συνυπάρχουν σε αυτά τα

συστήματα εξετάστηκε, με τη βοήθεια των σταθερών βιοαποδόμησης που υπολογίστηκαν μέσω πειραμάτων διαλείποντος έργου. Για τρεις ουσίες (OHBTH, BTR και XTR) οι κύριοι μηχανισμοί απομάκρυνσης ήταν η βιοαποδόμηση από την ενεργό ιλύ στον πρώτο αντιδραστήρα. Για τις CBTR και 5TTR η βιοαποδόμηση από τα δυο είδη βιομάζας ήταν περίπου ίδια και στους δυο αντιδραστήρες, ενώ η 4TTR απομακρύνθηκε κυρίως από το βιοφίλμ που σχηματίστηκε στον δεύτερο αντιδραστήρα. Η δημιουργία πιθανών παραπροϊόντων εξετάστηκε μέσω πειραμάτων διαλείποντος έργου. Συνολικά 22 ουσίες, πιθανοί μεταβολίτες ανιχνεύτηκαν ενώ η υδροξυλίωση, η οξείδωση και η μεθυλίωση ήταν οι κύριοι μηχανισμοί αντίδρασης. Συγκρίνοντας το υβριδικό σύστημα με τα συστήματα που εξετάστηκαν προηγουμένως, το τελευταίο συμπεριφέρθηκε όπως ένα σύστημα αντιδραστήρων κινούμενης κλίνης με βιοφορείς χαμηλής φόρτισης, ενώ αποδείχθηκε πιο αποτελεσματικό από συστήματα ενεργού ιλύος και κινούμενης κλίνης με βιοφορείς που λειτουργούσαν στον ίδιο υδραυλικό χρόνο παραμονής (HRT) και στις ίδιες συνθήκες φόρτισης.

Τα ακόλουθα κεφάλαια δομούν την παρούσα διατριβή: Το Κεφάλαιο 1 περιλαμβάνει μια σύντομη βιβλιογραφική ανασκόπηση που περιγράφει τις τεχνολογίες που χρησιμοποιήθηκαν, τις ουσίες που μελετήθηκαν και τέλος παρουσιάζει τους στόχους της εργασίας. Στο Κεφάλαιο 2 παρουσιάζεται η μεθοδολογία που ακολουθήθηκε και οι αναλυτικές μέθοδοι που χρησιμοποιήθηκαν. Στο Κεφάλαιο 3, παρουσιάζονται τα ευρήματα της μελέτης, ενώ στο Κεφάλαιο 4 συνοψίζονται τα βασικά συμπεράσματα και παρουσιάζονται προτάσεις για μελλοντική έρευνα. Στο τέλος της διατριβής παρατίθενται συμπληρωματικά στοιχεία καθώς και τρεις δημοσιεύσεις σε επιστημονικά περιοδικά που προέκυψαν από την παρούσα έρευνα.

Λέξεις κλειδιά

βενζοτριαζόλια (BTRs), βενζοθειαζόλια (BTHs), βιολογική επεξεργασία, υγρά απόβλητα, βιοαποδόμηση, προσρόφηση, ενεργός ιλύς, σύστημα αντιδραστήρων κινητού υποστρώματος βιοφορέων, βιοφίλμ, βιοφορείς

List of Publications

Papers in Scientific Journals

Paper A Mazioti, A.A., Stasinakis, A.S., Gatidou, G., Thomaidis, N.S., Andersen, H.R., 2015, Sorption and biodegradation of selected benzotriazoles and hydroxybenzothiazole in activated sludge and estimation of their fate during wastewater treatment. Chemosphere 131, 117-123

Paper B Mazioti, A.A., Stasinakis, A.S., Pantazi Y., Andersen, H.R., 2015, Biodegradation of benzotriazoles and hydroxy-benzothiazole in wastewater by activated sludge and moving bed biofilm reactor systems. Bioresource Technology 192, 627-635

Paper C Mazioti, A.A., Stasinakis, A.S., Psoma A.K., Thomaidis N.S., Andersen H.R., Hybrid Moving Bed Biofilm Reactor for the biodegradation of benzotriazoles and hydroxy-benzothiazole in wastewater Water Research (submitted for publication)

Papers in Scientific Conferences

Mazioti E.A., Stasinakis A.S. and Thomaidis N.S., Biodegradation and sorption of benzotriazoles and benzothiazoles in activated sludge systems. 13th International Conference on Environmental Science and Technology (CEST), 5-7 September 2013, Athens (oral presentation)

Mazioti A.A., Asimakopoulos A.G., Thomaidis N.S., Stasinakis A.S., Occurrence and fate of benzotriazoles during wastewater treatment, Singapore International Water Week - Water Convention Conference, 1-5 June 2014, Singapore (oral presentation)

Mazioti A.A, Stasinakis A.S., Andersen H.R, Pantazi Y., Study on the fate of BTRs and OHBTH in activated sludge and MBBR systems: Biodegradation kinetics and removal efficiencies, 25th Annual Meeting, Society of Environmental Toxicology and Chemistry (SETAC Europe), 3-7 May 2015, Barcelona (oral presentation)

Mazioti A.A, Stasinakis A.S., Andersen H.R, Pantazi Y., Removal of benzotriazoles and 2-OH-benzothiazole in lab-scale Moving Bed Bioreactors (MBBR) operated under different continuous-flow conditions, International Conference on Environmental Science and Technology (CEST), 3-5 September 2015, Rhodes (poster presentation)

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

2-Amino-BTH: 2-amino-benzothiazole

2-SH-BTH: 2-mercaptobenzothiazole

4TTR: 4-methyl-1H-benzotriazole

5TTR: 5-methy-1H-lbenzotriazole

AB: Aerobic Bioreactor

ACN: Acetonitrile

AS: Activated Sludge

BC1: Aerobic Bioreactor 1

BC2: Aerobic Bioreactor 2

BTHs: Benzothiazoles

BTH: Benzothiazole

BTRs: Benzotriazoles

BTR: 1H-benzotriazole

BTSA: 1,3-benzothiazole-2-sulfonic acid

CBTR: 5-chlorobenzotriazole

DAD: Diode Array Detector

COD: Chemical Oxygen Demand

DO: Dissolved Oxygen

EU: European Union

HMBBR: Hybrid Moving Bed Biofilm Reactor

HPLC: High Pressure Liquid Chromatography

HRT: Hydraulic Retention Time

k: first order biodegradation rate constant

k_{bio}: Biodegradation rate constant (normalized to the amount of biomass)

K_{OW}: Octanol-Water coefficient

K_d: Sorption coefficient

LOD: Limits of Detection

MBBR: Moving Bed Biofilm Reactor

MeOH: Methanol

MeSBTH: 2-Methylthio-Benzothiazole

MLSS: Mixed Liquor Suspended Solids

$NaNO_3$: Sodium Nitrate

NaN_3: Sodium Azide

OHBTH: 2-hydroxybenzothiazole

QToF-MS: Quadrupole-time-of-flight high-resolution mass spectrometer

REACH: Registration, Evaluation, Authorization and Restriction of Chemicals

RNA: Ribonucleic Acid

SRT: Sludge Retention Time

STP: Sewage Treatment Plant

SPE: Solid Phase Extraction

TAN: Total Ammonium Concentration

T: Temperature

TTR: Tolytriazole

TSS: Total Suspended Solids

UHPLC: Ultrahigh-performance liquid chromatography

UV: Ultraviolet

WWTP: Wastewater Treatment Plant

XTR: Xylytriazole

1. Literature Review

1.1. Biological Treatment of Wastewater

Treatment of wastewater is a moderately recent trend, and only in parts of the world where population has access to clean water for everyday use. The most ancient wastewater management system was discovered in Pakistan and is estimated to be constructed around 1500 BC, while other Roman and Hellenistic time cities (Rome, Pergamon ect.) are found to have constructed similar systems. The first designed wastewater reuse and management systems were applied in monasteries in Europe in the 12th and 13th century in order to make good use of water. The general confrontation in cities and organized communities was to dispose wastewater in an underground canal or open ditch to reach the closest river, while wastewater disposal was dealt as a problem and solutions were sought only in the 19th century due to major hygienic problems. Considering microbiology in this field, first observations of bacteria, protozoa and algae were made in the 17th century by Antoni van Leeuwenhoek. The first water sampling and quality analysis occurred in London and Berlin in the 1870s. The first suspicion that the clean-up of wastewater could be due to biological activity occurred at the same period (1870s) and became almost certain until the 1890s. From the 1860s the first tests for wastewater treatment were done with irrigation fields and were evolved until the 1900s to trickling filters. It was in 1913 that a new idea was introduced; to increase the concentration of aerobic bacteria by sludge sedimentation after aerating the sewage for several hours, to remove solid-free water and add sewage again. The first persons to observe an increase in sludge were Edward Arden and William T. Lockett and therefore the Activated Sludge (AS) process was born. A decade after this observation the first large scale plant was built in Germany (Wiesmann et al., 2006). Since then, biological sewage treatment processes have been widely applied and studied. Systems are mainly characterized according to the state in which biomass is encountered in the bioreactors. Based on this, they are usually divided to suspended growth and attached growth systems.

1.1.1. Activated Sludge (AS)

The most widely used suspended growth process is the AS process. It is used for biological treatment of both municipal and industrial wastewater. The name AS occurred from the involution of the

production of an activated mass of microorganisms capable of aerobic stabilization of organic matter in wastewater (Metcalf and Eddy, 2003). The basic AS process for organic load removal and nitrification consists of three components: a) a biological reactor where the microorganisms in the form of flocs are kept in suspension and aerated (aeration tank), b) a sedimentation tank or clarifier, and c) a recycle system for returning settled solids from clarifier to the reactor (Figure 1).

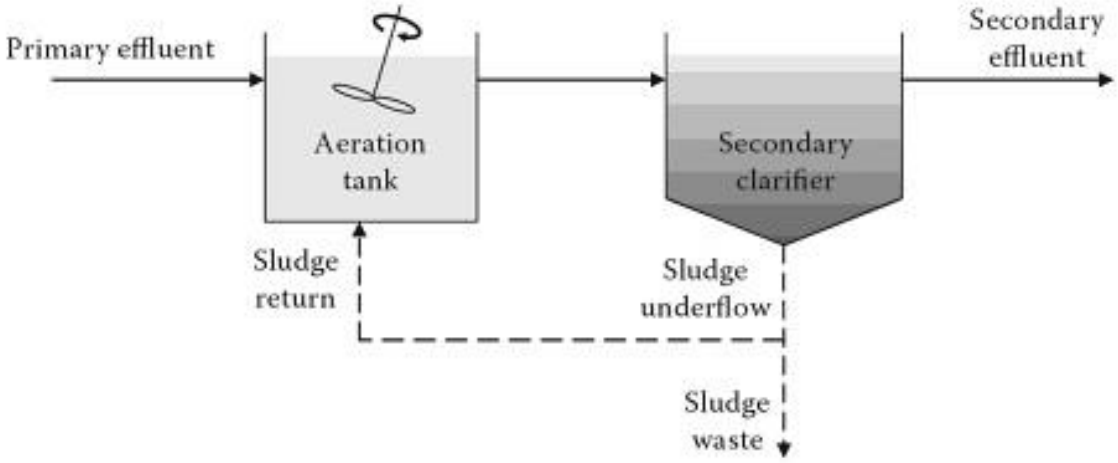


Figure 1: Activated Sludge (AS) process typical set-up for organic load removal and nitrification.

Wastewater flows continuously into the aeration tank or biological reactor, while air is provided in order to mix wastewater with microorganisms and to provide necessary oxygen for biological activity. The microorganisms degrade the organic matter in wastewater and produce cell mass and waste products. The mixed liquor is then driven to a second tank (secondary clariflier) where clarification of effluent and thickening of settled solids takes place. The clarified effluent is discharged for further treatment or disposal, while the thickened solids are periodically removed from the tank. A part of thickened solids is driven back to aeration tank (in order to maintain high concentration of AS), while the other is confronted as waste (Riffat, 2012). In such a system, nitrification can be simultaneously achieved under selected operating parameters. With the addition of extra bioreactors, full biological elimination of nitrogen can be achieved (via nitrification and denitrification) as well as elimination of phosphorus (Metcalf and Eddy, 2003). Many versions of the basic set-up presented in Figure 1 are applied and used, consisting this type of biological treatment the most known all over the world. A large body of knowledge exists, based on past and present research, on the design and operational

parameters, microbial communities, process models and removal capabilities of various pollutants (Riffat, 2012).

1.1.2. Moving Bed Biofilm Reactors

Moving Bed Biofilm Reactors (MBBRs) were introduced as a wastewater treatment technology during the late 1980s in Norway (Barwal and Chaudhary, 2014). They have been established as a simple, robust, flexible and compact technology, able to treat with high efficacy wastewater, occurring from many uses and activities (Jenkins and Sanders, 2012). There was growing interest the past decade considering their application, as MBBRs are an alternative option for wastewater treatment with many advantages, mainly concerning high quality water effluent at a generally low footprint (Rodgers and Zhan, 2003).

The process was developed when researchers in Lund University tried to find solutions for the biological treatment of difficult and toxic wastewater occurring from the pulp and paper industry. Studies evolved from small and pilot scale plants to full-scale installations and an enterprise (Anox AB) was set up in 1986 in Lund. Nowadays, more than one enterprises produce suspended biofilm carriers and commercially apply this type of treatment. However the dominating company is still AnoxKaldnes, part of the Veolia Water Technologies since 2007.

The applied technology is based on the trend of microorganisms to grow on surfaces and form biofilms. The biofilm grows on a media, on the protected inside surface, and at the same time the media (and biofilm) is transferred in all parts of the reactor. This media is called biocarrier and is usually made of thin and light plastic, of a certain shape (Figure 2). The biocarriers do circulate in all parts of the bioreactor, due to aeration or mechanical stirring, depending on the conditions desired in the reactor (Figure 3). The thickness of biofilm depends on many factors such as the design of the biocarrier, the available nutrients for microorganisms development as well as the time of residence of biocarriers in the reactor. An important factor that directly affects the growth of biofilm and the efficiency of a MBBR is the specific surface of the biocarriers, that can fluctuate from type to type between 200 $m^2\ m^{-3}$ (for model Natrix M2, AnoxKaldnes™) and 1200 $m^2\ m^{-3}$ (for model BiofilmChip M, AnoxKaldnes™) (Barwal and Chaudhary, 2014). Previous studies have focused on the design of biocarriers and on the optimization of their shape, having also in mind their life cycle and the cost of

production. It is worth saying that the life of biocarriers can vary from 10 to 30 years (Barwal and Chaudhary, 2014).

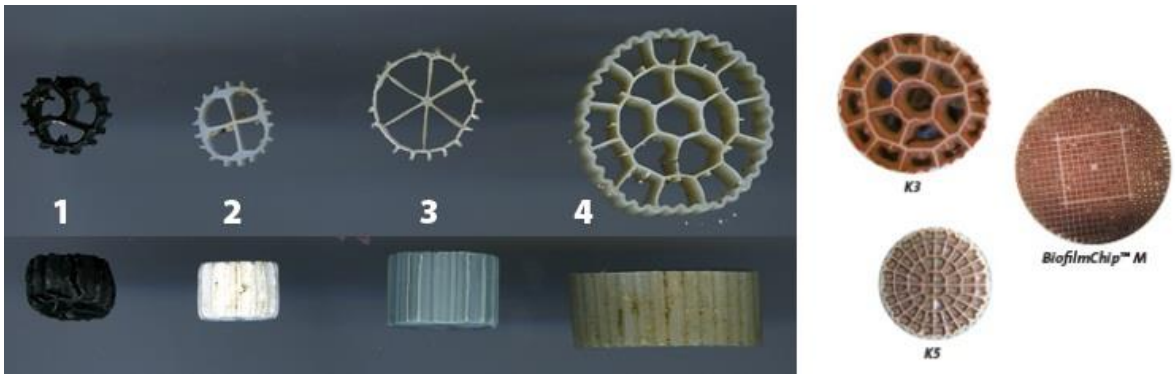


Figure 2: Types of biocarriers, (K3, K5 and BiofilmChip, developed by AnoxKaldnes™).

There are some factors that affect and determine an MBBR system. The redox conditions (oxic/anoxic) determine the type of biofilm that will be developed on carriers and as a result the biodegradation mechanisms that will dominate. In the case that a MBBR operates under aerobic conditions, the air flow proportion is an important parameter as it controls both the Dissolved Oxygen (DO) concentration in the reactor and the mixing conditions, which should be allowing the biocarriers to move in all parts of the MBBR. The biocarriers filling ratio is also crucial concerning operation, as it determines the concentration of attached biomass but also the amount of suspended solids (due to detachment of biofilm). It can vary from 30% to 70%, depending on the type of carrier and the MBBR design parameters. High filling ratio could lead to inadequate mixing and clogging and it is usually avoid. Di Trapani et al. (2008) reported that better Chemical Oxygen Demand (COD) removal was achieved at a filling ratio of 35%, compared to higher filling ratio (66%).

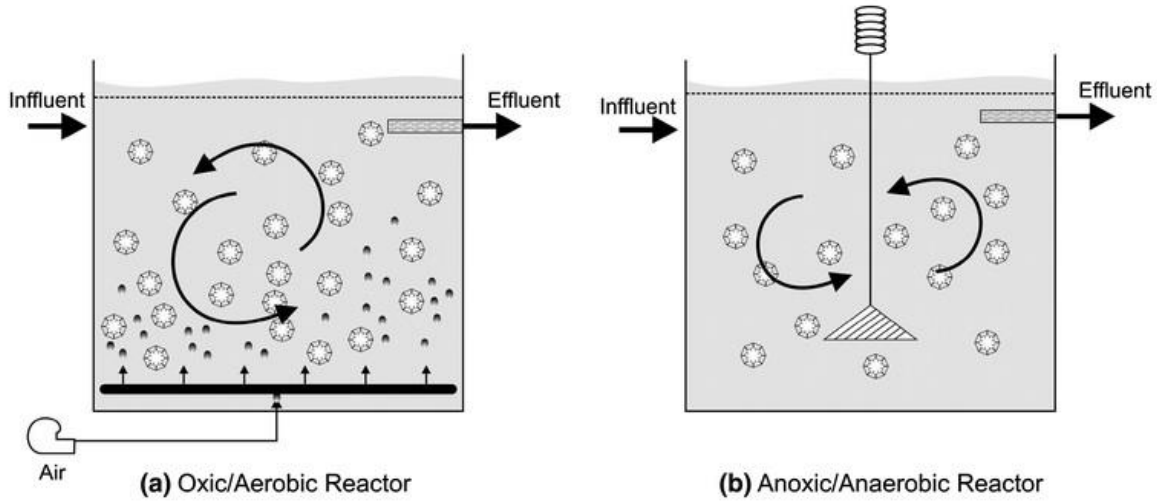


Figure 3: Schematic demonstration of Moving Bed Biofilm Reactors (MBBRs) operating under oxic and anoxic conditions (Barwal and Chaudhary, 2014).

An important advantage of the MBBRs is their increased nitrification capacity. The protected surface of area of biocarriers is ideal for the proliferation of nitrifying bacteria that have a relatively slow growth rate. As their growth rate is importantly affected by the water temperature, the MBBR technology has an important advantage over conventional AS in cold climate regions concerning nitrification efficiency (Barwal and Chaudhary, 2014). Total ammonium concentration (TAN) removal in an MBBR is influenced by many parameters, such as the organic load, DO concentration, TAN concentration, temperature (T) and pH (Figure 4). The organic loading is an important factor that can decrease significantly the TAN removal at a stable DO concentration when the rector is highly loaded (Rusten et al., 2006). Therefore low loaded conditions favor nitrification and usually occur in the last reactors when MBBRs are operated in series.

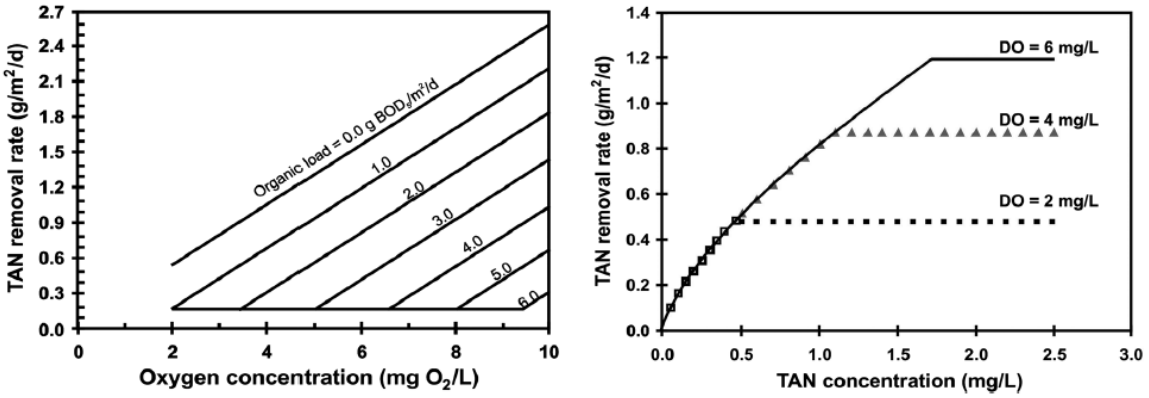


Figure 4: Influence of organic loading, dissolved oxygen (DO) and total ammonium concentration (TAN) concentration on TAN removal in MBBR systems (Rusten et al., 2006).

1.1.3. Hybrid Moving Bed Biofilm systems

Growing demand for more efficient wastewater treatment is leading to new technologies for treatment as well as improvement of the existing. The idea to combine the AS system with the MBBRs was introduced two decades ago for the first time in wastewater engineering (Randall et al., 1996; Gebara, 1999). The Hybrid Moving Bed Biofilm Reactor (HMBBR) is based on the combination of a typical AS system with a MBBR in which biofilm attached on biocarriers and AS flocs co-exist in the bioreactor, contributing to wastewater treatment. The main advantages of such a system, compared to the conventional AS system, are: a) the lower requirements for process volume, b) the increased nitrification capacity and c) the lower sludge load on the secondary clarifier (Di Trapani et al., 2013). Furthermore, the increased biomass concentration as well as the high microbial diversity assures satisfactory treatment, in many cases more efficient than conventional treatment systems (Mannina et al., 2007). Due to the above, HMBBR systems have been successfully used for upgrading existing AS systems (Mannina and Viviani, 2009; Di Trapani et al., 2011). The different properties and advantages or disadvantages that HMBBR present are not yet fully explored, as many combinations can be done regarding the conditions of treatment (aerobic/anaerobic), and the used biomass (combination or not of biofilm and AS, biocarrier type etc.) (Figure 5).

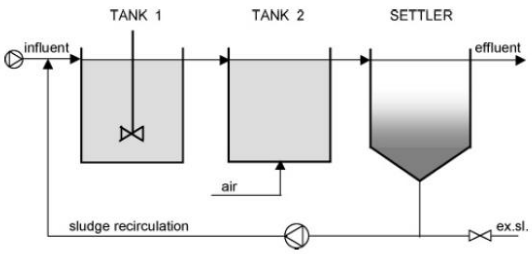


Figure 1. Scheme of the pilot plant without carriers.

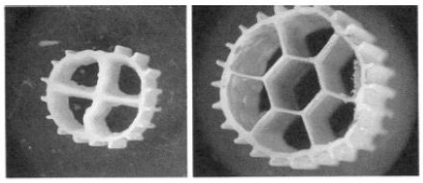


Figure 2. AnoxKaldnes carriers with biomass.

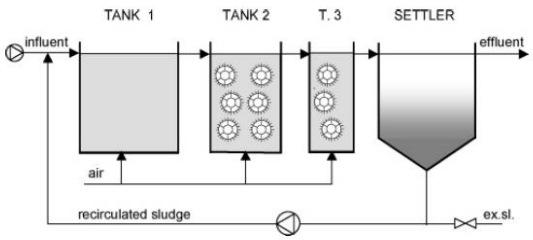


Figure 3. Pilot plant after the division of the aerated tank.

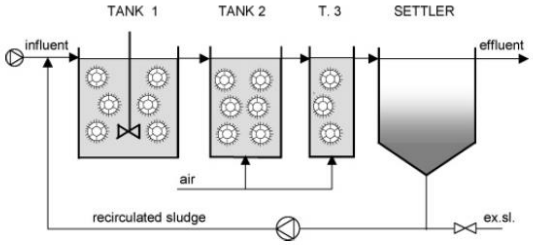


Figure 4. Pilot plant in its final configuration.

Figure 5: Several options for Hybrid Moving Bed Biofilm Reactor (HMBBR) concerning reactors organization and different types of carriers 1. anoxic and aerobic bioreactors with suspended biomass, 2. K1 and K2 biocarrier (from AnoxKaldnes), 3. aerobic bioreactors with suspended and attached biomass, 4. anoxic and aerobic bioreactors with suspended and attached biomass (Falletti and Conte, 2007).

1.1.4. Removal of Micropollutants from Wastewater during Biological Treatment

During biological treatment, two major mechanisms are responsible for micropollutants elimination: biodegradation/biotransformation and sorption (Verlicchi et al., 2012). The contribution of other mechanisms, such as volatilization and hydrolysis, to elimination of target compounds depends on their chemical properties, while the role of photodegradation being of minor importance due to the high concentration of biomass in bioreactors that does not allow significant light penetration.

Biodegradation of micropollutants generally occurs due to different mechanisms (Luo et al., 2014), a) single substrate growth of oligotrophic organisms, which mainly occur in surface water or sediment (Daughton and Ternes, 1999), b) co-metabolism, in which micropollutants are decomposed by enzymes generated for other primary substation degradation (for example, ammonia monooxygenase) and are not used as carbon and energy source for microbial growth, c) mixed substrate growth, whereas micropollutants are used as carbon and energy source and become mineralized (Vader et al., 2000). The biodegradability of a compound depends on the complexity of the compound (monocyclic or polycyclic) and its functional groups (for example halogen groups). On the other hand, sorption occurs by a) absorption, whereas interactions occur between the aliphatic and aromatic groups of a compound

and the lipophilic cell membrane of microorganisms as well as the fat fractions of sludge, and b) adsorption, involving the electrostatic interactions of the positively charged groups with the negatively charged surfaces of the microorganisms and sludge (Ternes et al., 2004; Luo et al., 2014). Sorption of micropollutants to solids depends strongly on the hydrophobicity of each compound (Luo et al., 2014), whereas the acidity determined by the functional group of a compound can play an important role on the chemisorption or/and electrostatic adsorption of micropollutants (Schäfer et al., 2011). For compounds that have a sorption coefficient (K_d) lower than 300 L Kg^{-1}, sorption to sludge is considered insignificant. In general, compounds that tend to be sorbed to organic matter are expected to be eliminated at some extend by AS (Luo et al., 2014).

Some parameters applied in a Sewage Treatment Plant (STP) may influence the micropollutants removal. The Sludge Retention Time (SRT) is responsible for the size and the diversity of the microbial community and is proposed to enhance some micropollutants removal when higher (Fernandez-Fontaina et al., 2012; Suárez et al., 2010). A high SRT facilitates the development of slow-growing bacteria, such as nitrifying bacteria, whereas co-metabolism using ammonium monooxydase enzyme is a possible pathway for micropollutants degradation. Despite the cases in which researchers found that a high STR enhanced biodegradation of micropollutants, there are also studies that found no differences in removal, even with high SRTs (Joss et al., 2005; Santos et al., 2009; Stasinakis et al., 2020). Another parameter influencing biodegradation is the Hydraulic Retention Time (HRT), which is actually the available time for interaction of micropollutants and microorganisms. The compounds with slow kinetics are expected to be less effectively biodegraded at short HRTs (Luo et al., 2014). The redox conditions (oxic/anoxic) may also influence biodegradation, having an effect on biodiversity of the microbial flora and the general sludge characteristics (Göbel et al., 2007). Finally, wastewater characteristics such as pH and temperature may influence removal. The acidity or alkalinity of the aqueous environment can influence both the physiology of microorganisms and the solubility of micropollutants present in wastewater (Cirja et al., 2008).

It is obvious from the above that the biodegradation of micropollutants is a complicated task, with many parameters interfering and influencing this process. This may create difficulties considering their study but also gives space for optimization of the process in order to fully take advantage of this step in Waste Water Treatment Plants (WWTPs), for achieving maximum removal of micropollutants before the application of further treatment methods.

Though biofilms may be a key technology for the removal of toxic and emerging pollutants (Borghei et al., 2004; Edwards and Kjellerup, 2013), so far, only few studies have examined the removal of micropollutants using MBBRs and HMBBRs. Specifically, Falås et al. (2012) investigated pharmaceuticals degradation and calculated removal rate constants in batch experiments with carriers that had been collected from different full-scale STPs, while in a recent work the same authors investigated the removal of 20 micropollutants by monitoring a full scale hybrid biofilm/AS plant (Falås et al., 2013). In another study, the removal of three hormones was examined by early-stage biofilm in batch tests (Khan et al., 2013), while Luo et al. (2014) operated a bench-scale MBBR system with polyurethane sponge carriers in order to determine various micropollutants removal. Finally, Accinelli et al. (2012) examined the removal of bisphenol-A, atrazine and oseltamivir with bioplastic carriers inoculated with specific bacterial strains. Escolà Casas et al. (2015) investigated the removal of 26 pharmaceuticals in hospital wastewater by a 4 staged pilot treatment plant consisting of AS, HMBBR and MBBR reactors in series and reported biodegradation kinetics in different bioreactors. Finally, Sfaelou et al. (2015) recently examined the effects and removal of phenanthrene in sequencing batch reactors containing AS and biocarriers. Limited information is available for the role of organic loading (Ahmadi et al., 2015) and the contribution of different reactors in series on micropollutants removal in a MBBR system. Therefore, information focusing on the biodegradation of micropollutants in MBBR and HMBBR systems is valuable.

1.2. Benzotriazoles and Benzothiazoles

Benzotriazoles (BTRs) and Benzothiazoles (BTHs) are two classes of compounds, included in the large category of emerging contaminants (Stasinakis, 2012). No legislation is yet implied by the European Union (EU) about concentration limits when disposed through treated wastewater to the environment. On the other hand, European Chemicals legislation concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) was entered into force in 2007 and aims to ensure a high level of protection of human health and the environment (regulation EC 1907/2006). When REACH will be fully into force, companies handling, manufacturing or importing large quantities of chemicals will have to register these compounds, in order to control the circulation of chemicals through their main sources.

1.2.1. Properties, Uses and Toxicity

1.2.1.1. Benzotriazoles

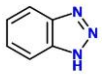
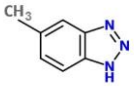
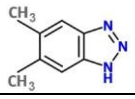
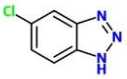
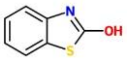
BTRs consist of a benzene ring fused with a triazole ring; for most compounds the five-membered ring can exist in tautomers (Table 1). These compounds are highly soluble in water, slightly basic and highly polar (Weiss et al., 2006; Reemtsma et al., 2010), leading to their weak tendency to sorb onto organic matter (Table 1). They are used in a large variety of applications, at a household as well as an industrial level (Jia et al., 2007; Farré et al, 2008), which results in high amount of these chemicals being handled annually. They are mainly used as corrosion inhibitors in metal finishing industry and especially for the protection of copper and its alloys. Furthermore they are used in de-icing fluids, in hydraulic fluids, in cooling fluids, in photography as restrainers and in dishwashing detergents (Reemtsma et al., 2010; Kiss and Fries, 2012; Loi et al., 2013; Cantwell et al., 2015). Airports in cold areas are considered an important source for direct environmental disposal of these compounds, where deicing fluids are used in large quantities on aircrafts (Breedveld et al., 2003; Cancilla et al., 2003). Considering their impact on health, it was described in an older report that BTRs could be able to affect the nervous and endocrine system and inhibit the formation of proteins, enzymes and Ribonucleic Acid (RNA) in mammals, due to the similarities they present with compounds such as adenine and guanine (USEPA, 1977). Furthermore, Castro et al. (2005) characterized BTRs as possible carcinogenic compounds, while benzotriazole (BTR) is considered to be an endocrine disrupting compound (Kadar et al., 2010). Concerning their toxicity, concentrations of BTRs higher than 100 mg L^{-1} can cause acute toxicity to prokaryotic and eukaryotic organisms (Pillard et al., 2001) and concentrations up to some mg L^{-1} can cause acute and chronic toxicity to aquatic organisms (Seeland et al., 2012). When examining benzotriazole ultraviolet stabilizers, Kim et al. (2011) indentified concentration of some hundreds of ng g^{-1} in fish tissues, highlighting that these compounds accumulate through benthic food chain in fish (Kim et al., 2011). Further research is needed for the evaluation of toxic effects that could have on living organisms.

1.2.1.2. Benzothiazoles

BTHs consist of a benzene ring fused with a thiazole ring (Table 1). They also present high polarity, due to low octanol-water coefficient (K_{ow}), as well as high water solubility, lower than for BTRs (Bahnmüller et al., 2015). BTHs rarely occur as natural products and they are mainly used in industrial

applications but also as additives in drugs, biocides and food flavors. In industry, they are used as vulcanization accelerators in rubber production, as slimicides in paper and pulp processing and as corrosion inhibitors in cooling fluids (Wever and Verachtert, 1997; Ni et al., 2008; Vigan, 2011; Loi et al., 2013). They are considered toxic substances but only at concentrations higher than environmentally encountered (Herrero et al., 2014). De Wever et al. (1997) reported growth inhibition of bacteria and yeast when BTHs were present at concentrations in the range of decades of mg L^{-1}. Similar to BTRs, further research is needed considering their toxicity.

Table 1. Target compounds that were analyzed in the present study.

Compound	Molecular Formula	Chemical Structure	M.W.	$LogK_{ow}$	pKa
1H-benzotriazole (BTR)	$C_6H_5N_3$		119.12	1.23[2]	8.37[1]
4-Methyl-1H-benzoriazole (4TTR)	$C_7H_7N_3$		133.15	1.89[2]	8.5[2]
5-Methyl-1H-benzoriazole (5TTR)	$C_7H_7N_3$		133.15	1.89[2]	8.5[2]
5,6-dimethyl-1H-benzotriazole or xylytriazole (5,6DMTR or XTR)	$C_8H_9N_3$		147.18	2.06[5]	9.28[5]
5-Chlorobenzotriazole (CBTR)	$C_6H_4ClN_3$		153.57	2.17[6]	7.5/7.7[6]
2-hydroxybenzothiazole (OHBTH)	C_7H_5NSO		151.2	1.76[3]	8.65[4]

[1]Yang et al., 2011; [2]Hart et al., 2004; [3]Leerdam et al., 2009; [4]Andreozzi et al., 2001; [5]http://www.chemicaldictionary.org/dic/5/56-Dimethyl-1H-benzotriazole_1893.html; [6] Liu et al., 2012.

1.2.2. Occurrence in the Environment

Both groups of BTRs and BTHs are frequently detected in the environment (surface water, underground water and drinking water), as a consequence of their partial removal from wastewater. The detected concentrations in water vary from a few ng L^{-1} up to some hundreds of ng L^{-1}, while high concentrations in the range of mg L^{-1} have been observed in surface waters close to airports, due to the

extended use of de-icing fluids (Cancilla et al., 1998). On the other hand, very low concentrations in the range of some ng g^{-1} are detected in sediments and sludge (Careghini et al., 2013; Alotaibi et al., 2015). A revealing study that focuses on the existence of BTRs and BTHs in human urine proves that humans are exposed to these compounds (Asimakopoulos et al., 2013a).

1.2.2.1. Benzotriazoles

A recent European study on polar organic micropollutants in river waters examined 100 rivers in 27 countries (Loos et al., 2009). This research revealed that two BTRs are frequently detected and at high concentrations, among 35 micropollutants. 1H-benzotriazole (BTR) and Tolytriazole (TTR) were among the most frequently detected compounds, identified in almost all water samples (Figure 6). Their median concentration was again among the highest, 226 ng L^{-1} for BTR and 140 ng L^{-1} for TTR (Figure 7). Furthermore, high maximum concentration was observed for these two compounds, 20 μg L^{-1} for TTR and 8 μg L^{-1} for BTR (Loos et al., 2009).

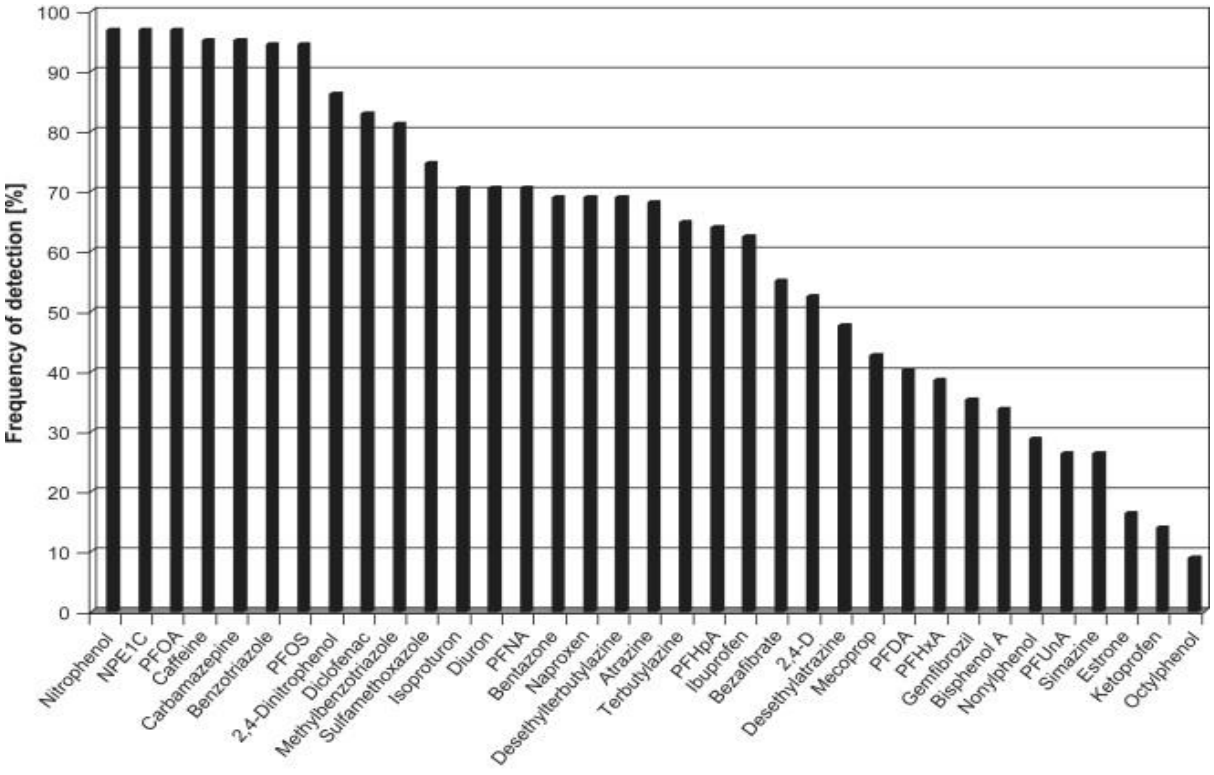


Figure 6: Polar organic micropollutants frequency of detection (%) in European surface waters (Loos et al., 2009).

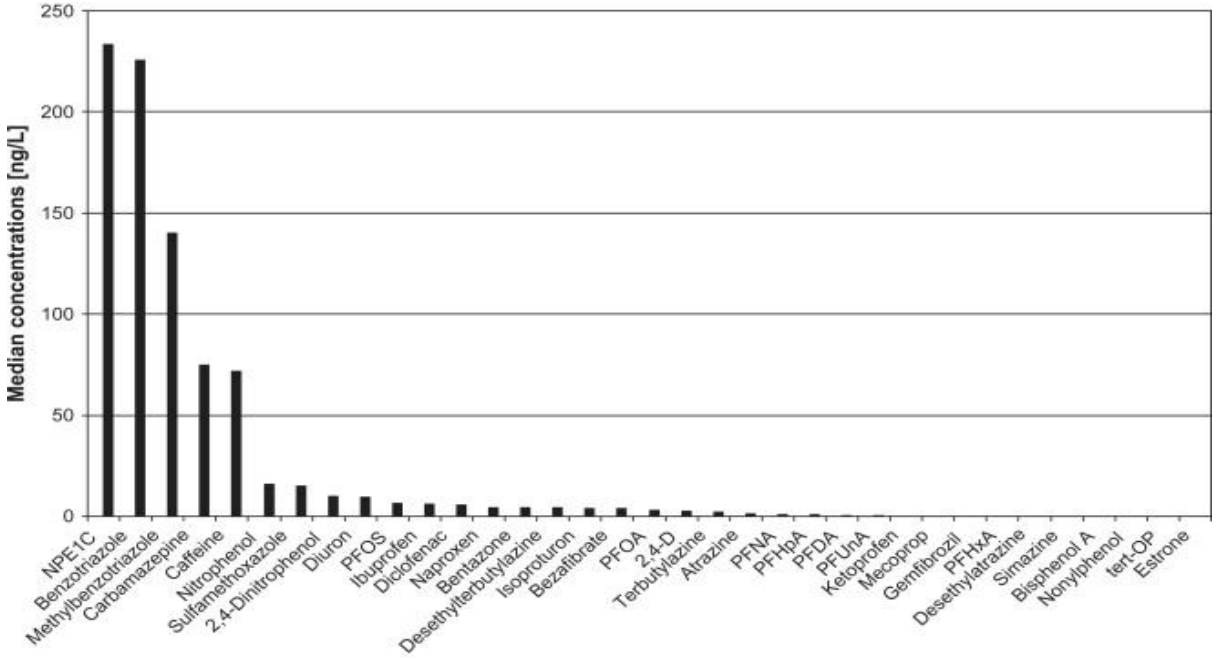


Figure 7: Polar organic micropollutants median concentrations (ng L^{-1}) in European surface waters (Loos et al., 2009).

Similar results for river concentrations of these compounds have been obtained by many research groups all over the world. Considering European countries, in Germany, BTR and TTR are frequently detected in river sites with median concentrations in the range of some decades to some thousands of ng L^{-1} (Kiss and Fries, 2009; Reemtsma et al., 2010; Fries et al., 2011a). The same range for median concentrations is observed for BTR and TTR in rivers in Switzerland (Giger et al., 2006; Voutsa et al., 2006), in rivers in Spain (Gorga et al., 2015) and in a river in the United Kingdom (Janna et al., 2011). Records also exist for the detection of various BTRs in rivers (water, sediments and estuary) in North America (Hartmann et al., 2005; Hagedorn et al., 2013) and Asia (Kameda et al., 2011; Zhang et al., 2011). Concentrations of BTRs in seawater are lower, due to the high dilution factor of river water. Only some ng L^{-1} have been detected in all cases (Wolschke et al., 2011; Loos et al., 2013). In groundwater different concentrations have been reported for BTRs, varying from some ng L^{-1} to some thousands of ng L^{-1} (Loos et al., 2010; Reh et al., 2013).

1.2.2.2. Benzothiazoles

BTHs were monitored for the first time in the late 1970s in river and drinking water (De Wever et al., 2001; Brownlee et al., 1992). However, less monitoring studies are available than BTRs, with BTHs

presence being more frequently examined in wastewater and not in surface waters (Herrero et al., 2014). Bester et al. (1997) detected some ng L^{-1} of BTHs in river water and sea samples in Germany. Higher concentrations were determined in China in riverine runoff samples, with BTHs being present at some thousands of ng L^{-1} (Ni et al., 2008). On the other hand, low frequency of detection has been reported for some BTHs in surface waters of North China (Kong et al., 2015).

1.2.3. Occurrence and Fate during Biological Wastewater Treatment

Both BTRs and BTHs are not completely removed in STPs (Domínguez et al., 2012). Differences on their removal rates are observed in monitoring studies in different STPs all over the world, indicating that STP's operational parameters and other factors affect their degradation and removal (Kloepfer et al., 2005; Liu et al., 2012; Stasinakis et al., 2012).

1.2.3.1. Benzotriazoles

Concerning BTRs, the most frequently examined compounds are BTR and TTR, a mixture of two isomers, 4methyl-1H-benzotriazole (4TTR) and 5methyl-1H-benzotriazole (5TTR). These two compounds are detected in raw sewage at higher concentrations compared to other BTRs and the major part of studies concerning BTRs have focused on them (Nödler et al., 2010; Liu et al., 2012). Table 2 summarizes the concentrations of BTRs that were detected in raw and treated sewage in STPs all over the world (Herrero et al., 2014). Their concentration in raw sewage depends on the source of the sewage and sometimes can reach some decades or even hundreds of μg L^{-1} in highly polluted wastewater (Jover et al., 2009; Matamoros et al., 2010a).

Table 2: Occurrence of BTRs in STPs (raw and treated sewage) (Herrero et al., 2014)

	Raw sewage (μg/L)	Treated sewage (μg/L)	Country	References
BTR	5-7	2-3	Australia	Liu et al., 2011; Liu et al., 2012
	1-44	1-10	Germany	Weiss et al., 2005; Weiss et al., 2006; Nödler et al., 2010; Reemtsma et al., 2010; Dominguez et al., 2012

	Raw sewage (µg/L)	Treated sewage (µg/L)	Country	References
	0.5-3	0.01-0.5	Greece	Asimakopoulos et al., 2013; Stasinakis et al., 2013
	0.5-210	0.06-8	Spain	Jover et al., 2009; Matamoros et al., 2010a; Matamoros et al., 2010b; Carpinteiro et al., 2012; Herrero et al., 2013
	13-75	11-100	Switzerland	Voutsa et al.,2006; Giger et al., 2006
4TTR	2-6	1-2	Germany	Weiss et al., 2005; Weiss et al., 2006; Reemtsma et al., 2010
	<0.06-11	0.04-7	Spain	Jover et al., 2009; Herrero et al., 2013; Matamoros et al., 2010; Pena et al., 2012
5TTR	5-8	0.4-0.9	Australia	Liu et al., 2011; Liu et al., 2012
	1-5	0.5-2	Germany	Weiss et al., 2005; Weiss et al., 2006; Reemtsma et al., 2010; Dominguez et al., 2012
	<0.06-5	0.02-17	Spain	Jover et al., 2009; Herrero et al., 2013; Matamoros et al., 2010; Pena et al., 2012
TTR	3-16	0.3-6	Greece	Asimakopoulos et al., 2013b; Stasinakis et al., 2013
	0.4-91	0.9	Spain	Carpinteiro et al., 2012
	0.2-6	0.1-4	Switzerland	Voutsa et al.,2006; Giger et al., 2006

	Raw sewage (μg/L)	Treated sewage (μg/L)	Country	References
XTR	0.9-2	0.1-0.2	Australia	Liu et al., 2011; Liu et al., 2012
	0.02	0.01	Germany	Weiss et al., 2005
	<0.03	<0.03	Greece	Stasinakis et al., 2013
	<0.01-14	<0.005	Spain	Herrero et al., 2013
CBTR	0.6-2	0.08-0.3	Australia	Liu et al., 2011; Liu et al., 2012
	<0.01-14	<0.005	Spain	Herrero et al., 2013; Pena et al., 2012
OHBTR	0.1-0.5	<0.2	Greece	Asimakopoulos et al., 2013b; Stasinakis et al., 2013

According to monitoring studies, these compounds are partially removed during wastewater treatment. Liu et al. (2012) examined removal in a municipal STP in Australia and reported that BTR was eliminated from 7% to 27% due to biological activity. Furthermore they reported that during the whole process, 5TTR and xylytriazole (XTR) were eliminated at rates higher than 87% while 5-chlorobenzotriazole (CBTR) was eliminated by 56%. Voutsa et al. (2006) examined the elimination of BTR and TTR in 10 STPs in Switzerland and discovered high fluctuations in removal from plant to plant. The removal rates varied from 3% to 62% for BTR and from 18% to 74% for TTR. Monitoring studies were also conducted in Germany by Reemtsma and Weiss (2010 and 2006). More specifically, Weiss et al. (2006) investigated the removal of BTR, 4TTR and 5TTR in a STP through sampling campaigns that lasted more than one year. They reported removal for BTR varying from 5% to 60%, no

removal was observed for 4TTR, while 5TTR was eliminated by 11%. On the other hand, Reemtsma et al. (2010) examined 4 STPs of Berlin and mentioned 29% to 58% removal for BTR; 34% removal for 4TTR (but only in one STP) and finally 19% to 69% removal for 5TTR. In an earlier study in STPs of Germany, Belgium, Spain and Austria, Reemtsma et al. (2006) had mentioned 35% removal for BTR and 10% for TTR. In Spain, Matamoros et al. (2010a) observed 60% removal for BTR in two STPs.

So far, some laboratory studies have focused on the biodegradation of selected BTRs by AS (Liu et al., 2011; Fålas et al., 2012; Fålas et al., 2013; Huntscha et al., 2014). More specifically, in experiments with AS and initial concentration of target compounds equal to 1 mg L^{-1}, Liu et al. (2011) studied the biodegradation potential of BTR, 5TTR and CBTR under aerobic conditions and proposed their biotransformation pathways. In a recent study, Huntscha et al. (2014) investigated the biotransformation of BTR, 4TTR, and 5TTR under aerobic conditions (initial concentrations: 0.5-2.4 mg L^{-1}), determined their half-lives and identified the major biotransformation products. Finally, Herzog et al. (2014a, b) studied the removal efficiency of BTR, 4TTR and 5TTR under different experimental conditions at initial concentrations ranging between 0.2 and 34 mg L^{-1}, and reported that sludge acclimatization enhanced biodegradation of some compounds.

1.2.3.2. Benzothiazoles

Concerning BTHs, they are generally detected at lower concentrations in STPs compared to BTRs and benzothiazole (BTH) is the most frequently detected compound (Table 3).

Table 3: Occurrence of BTHs in STPs (raw and treated sewage) (Herrero et al., 2014)

	Raw sewage (μg/L)	Treated sewage (μg/L)	Country	Citation
BTH	0.4-1	0.07-12	Germany	Reemtsma et al., 2000; Kloepfer et al., 2005; Wick et al., 2010; Fries et al., 2011b; Dominguez et al., 2012
	0.5-1	<0.05-0.6	Greece	Asimakopoulos et al., 2013b; Stasinakis et al., 2013
	0.2-1	<0.1-3	Spain	Jover et al., 2009; Matamoros et al., 2010a; Matamoros et al., 2010b; Carpinteiro et al., 2012; Herrero et al., 2013

	Raw sewage (μg/L)	Treated sewage (μg/L)	Country	Citation
OHBTH	0.2-0.8	0.1-0.5	Germany	Reemtsma et al., 2000; Kloepfer et al., 2004; Kloepfer et al., 2005; Wick et al., 2010; Dominguez et al., 2012
	0.3-0.9	0.09-0.5	Greece	Asimakopoulos et al., 2013b; Stasinakis et al., 2013
	0.1-11	0.005-3	Spain	Céspedes et al., 2006; Jover et al., 2009; Matamoros et al., 2010a; Carpinteiro et al., 2012; Herrero et al., 2013
MeSBTH	0.2-0.4	0.2-13	Germany	Reemtsma et al., 2000; Kloepfer et al., 2004; Kloepfer et al., 2005; Wick et al., 2010
	0.2-4	0.04-0.4	Greece	Asimakopoulos et al., 2013b; Stasinakis et al., 2013
	0.1-13	0.06-1	Spain	Céspedes et al., 2006; Jover et al., 2009; Matamoros et al., 2010a; Matamoros et al., 2010b; Carpinteiro et al., 2012; Pena et al., 2012; Herrero et al., 2013

Though one study reported average removal of 87% for BTHs in sewage (Kloepfer et al., 2005), these compounds are partially removed during wastewater treatment and their removal efficiencies ranged between 20% to 80% for BTH; 50% to 60% for 2-hydroxybenzothiazole (OHBTH); higher than 95% for 1,3-benzothiazole-2-sulfonic acid (BTSA); higher than 75% for 2-amino-benzothiazole (2-Amino-BTH) and 10% for 2-mercaptobenzothiazole (2-SH-BTH) (Reemtsma et al., 2006; Matamoros et al., 2010a). Only few studies have focused on their biological degradation, while there is no information on their biodegradation kinetics with AS. Wever and Verachtert (1997) have investigated the potential to remove BTHs from industrial wastewater with biological degradation and focused on the isolation of bacteria strains, able to degrade these compounds. On the other hand, Bester and Schäfer (2009) examined the potential to remove BTHs with an activated soil filter (bio-filter) as a solution for the elimination of micropollutants from storm and waste water. Finally, Schoenerklee et al. (2010) developed a biokinetic model and estimated BTSAs removal from wastewater.

So far, no data is available for the fate of XTR and OHBTH in activated sludge process, concerning kinetics describing their biodegradation. Furthermore, though biodegradation of micropollutants during activated sludge process is affected by factors such as the existence of aerobic and anoxic conditions, the sludge residence time (SRT) and the presence of supplementary organic substrate (Joss et al., 2004; Stasinakis et al., 2009; Falås et al., 2012; Vasileiadou et al., 2014) there is lack of knowledge for the role of these parameters on BTRs and BTHs elimination. Moreover, though sorption is not expected to be a major removing mechanism, there is limited data for the sorption potential of BTRs and BTHs to sludge (Stasinakis et al., 2013), as well as for the contribution of biodegradation and sorption on their removal from STPs.

On the other hand, there are no studies examining the removal of BTRs and BTHs during secondary treatment, in lab-scale continuous flow systems. Matamoros et al. (2010a) tested the removal of some BTRs and BTHs in constructed wetlands that accepted secondary treated sewage. The HRT was approximately 1 month and the influent concentration of target compounds in the range of some $\mu g\ L^{-1}$. They observed removal rates of approximately 50% for BTR, 70% for 4TTR, 50% for 5TTR, 80% for BTH and 45% for OHBTH. As described in the last paragraph of section 1.1.4. there are few studies focusing on the comparison of lab scale AS, MBBR and HMBBR systems for the removal of micropollutants. Concerning removal of BTRs with biofilms, only Falås et al. (2013) has published information for BTR and TTR biodegradation constants, while there is no other available information for the comparison of target compounds removal with attached and suspended biomass.

1.3. Novelty of the thesis

Based on the available literature data reported above, there is limited (or no) information on the following topics concerning the fate of BTRs and BTHs during biological wastewater treatment.

There is only one study presenting information for the sorption constants of target BTRs and BTHs onto AS (Stasinakis et al., 2013). In that study, constants have been calculated by monitoring a full-scale STP. So far, no laboratory studies have been conducted for estimating target compounds sorption capacity onto AS.

Beside the fact that the effect of parameters such as the SRT, the organic load and the redox conditions has been studied in the past for several groups of micropollutants (Joss et al., 2004; Stasinakis et al.,

2009; Falås et al., 2012; Vasileiadou et al., 2014), so far there is no information for the role of these factors on the biodegradation kinetics of BTRs and BTHs in AS systems.

The elimination and fate of BTRs and BTHs has never been studied in continuous-flow AS, MBBR and HMBBR systems. So far, no comparison on the removal efficiency of organic micropollutants in such systems has been conducted.

The biodegradation kinetics of BTRs and BTHs have never been calculated for attached biomass (biofilm), while there is no information for the effect of organic substrate on the kinetics.

There is no information for the TPs of target compounds in HMBBR systems.

1.4. Aims and Outline of the Thesis

The aim of this study was to investigate the comportment of six compounds contained in the group of BTRs and BTHs during biological treatment. More specifically, BTR, CBTR, XTR, 4TTR, 5TTR and OHBTH were studied (Table 1). The main and specific objectives as well as the outline of this PhD Thesis are reported below.

Main Objectives

1. Study of the fate of BTRs and BTHs during biological wastewater treatment and investigation of the role of biodegradation and sorption on their removal.
2. Comparison of BTRs and BTHs removal efficiency in different biological treatment systems.

Specific objectives

1. Investigation on the role of supplementary organic substrate and SRT on BTRs and BTHs biodegradation kinetics.
2. Determination of the more suitable redox conditions (oxic/anoxic) for the biodegradation of target compounds.
3. Examination of the sorption capacity of BTRs and BTHs onto AS.
4. Evaluation of the biodegradation potential of suspended (activated sludge) and attached biomass (biofilm grown on carriers) on target compounds.
5. Study of the role of organic loading in the performance of biological wastewater treatment systems for the removal of target micropollutants.

6. Identification of the BTRs and BTHs biotransformation products produced in hybrid wastewater treatment systems.
7. Utilization of experimentally obtained data for the prediction of target compounds fate during sewage treatment.

To achieve these goals, the following three studies were conducted and the results were published in **Papers A to C**:

A. Investigation of BTRs and BTHs sorption and biodegradation in activated sludge: During this study, sorption coefficients were calculated for each compound through batch experiments with AS. The kinetics describing biodegradation of target compounds by AS were examined and biodegradation constants were calculated. Furthermore, the influence of redox conditions, SRT and presence of organic substrate on biodegradation constants was examined. The information obtained was used in order to estimate the fate of the compounds in large scale STPs (**Paper A**).

B. Comparison of BTRs and BTHs biodegradation in AS and MBBR systems: An AS and a MBBR lab-scale system were operated in parallel and the elimination of target compounds was investigated. The biodegradation of BTRs and BTHs by suspended (activated sludge) and attached biomass (biofilm on carriers) was studied, while the role of organic loading in the removal of target compounds was investigated (**Paper B**).

C. Study of BTRs and BTHs fate and removal in an HMBBR system: An HMBBR lab-scale system was used and the removal efficiency of target compounds was compared with that observed in previously used AS and MBBR systems. The contribution of different types of biomass existing in the HMBBR was examined concerning the biodegradation of the BTRs and BTHs. The formation of by-products was also investigated through batch experiments (**Paper C**).

2. Experimental and Analytical Methods

2.1. Experimental Procedure

All the experimental procedure described in paragraphs 2.1.2 and 2.1.3 was conducted in the Water and Air Quality Laboratory of the Department of Environment, University of the Aegean.

2.1.1. Biomass and Sewage Sampling

Three full-scale STPs were involved in this study (Figure 8 and Table 4).

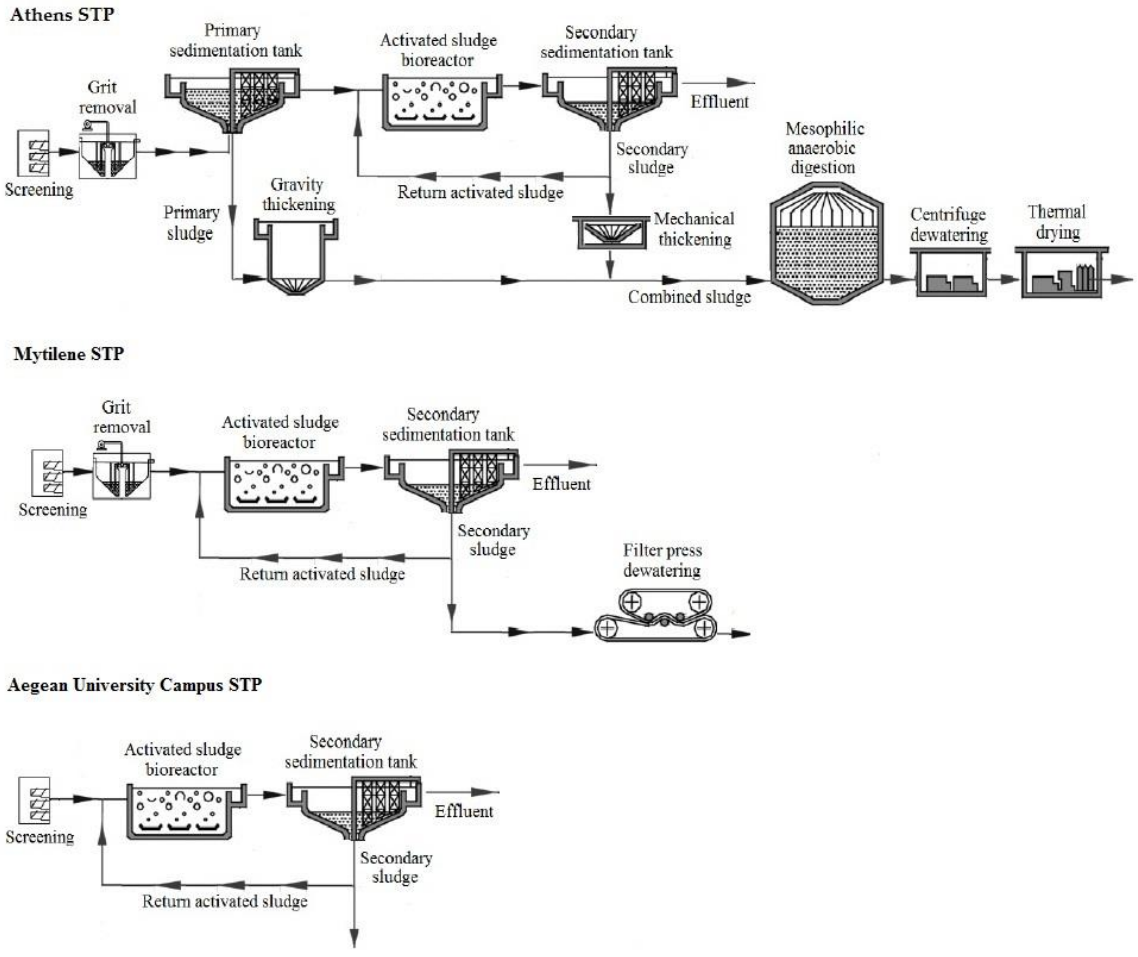


Figure 8: Schematic representation of the Sewage Treatment Plants (STPs) involved in this study.

Athens and Mytilene STPs operating parameters were used for modeling and predicting the potential of these two plants on target micropollutants removal (**Paper A**). Mytilene STP was used for AS collection in sorption and biodegradation batch experiments as well as for the inoculation of lab scale systems (**Paper A, B, C**). AS was also collected from Athens STP for conducting batch biodegradation experiments (**Paper A**). Furthermore, Aegean University Campus STP was used as a source for raw sewage collection.

Athens STP has an average treatment capacity of 650,000 m^3 per day, with inflow sewage occurring at 80% from domestic use and 20% from industrial use. The STP has the following treatment stages: pretreatment (screening, grit removal), primary sedimentation, AS process with biological nitrogen and phosphorus removal and secondary sedimentation. The HRT in AS bioreactors is approximately 9 hours, while the SRT is 8 days.

Mytilene STP has a treatment capacity of 4,500 m^3 per day with inflow sewage occurring only from domestic uses. The STP has the following treatment stages: pretreatment (screening, grit removal), AS process with biological nitrogen and phosphorus removal and chlorination. The HRT in AS bioreactors is approximately 24 hours, while the SRT is 18 days. Further information for the operating parameters of both STPs can be found in previous papers (Stasinakis et al., 2008; Samaras et al. 2013).

The University Campus STP treats sewage that occurs from domestic use. The average daily treatment capacity is 50 m^3 per day. This STP has a pretreatment stage (screening) and applied AS process for biological treatment of sewage. The HRT in the AS bioreactor is approximately 30 h. Information on the raw sewage quality parameters can be found in supplementary materials of **Paper B** and **Paper C**.

Table 4: Operating parameters of STPs examined in this study.

STP	Flowrate (m^3 d^{-1})	Screening	Grit Removal	Primary Sedimentation	Activated Sludge Bioreactors		Sludge Anaerobic Digestion
Athens[1]	750000	YES	YES	YES	HRT = 10 h	SRT = 8 d	YES
Mytilene[1]	7000	YES	YES	NO	HRT = 22 h	SRT = 18 d	NO
University Campus[2]	50	YES	NO	NO	HRT = 30 h	SRT = 40 d	NO

[1]Biological N and P removal during activated sludge process using anaerobic, anoxic and aerobic reactors in series; [2]Removal of BOD and nitrification using only aerobic reactor

2.1.2. Sorption and Biodegradation Batch Experiments

Concerning sorption experiments, AS collected from Mytilene STP was used as an adsorbent material. Sludge preparation occurred according to the method applied by Andersen et al. (2005) and Hörsing et al. (2011). The pretreatment of sludge included washing with tap water (3 times), centrifugation and freezing at -18 ^{o}C for 24 hours. Furthermore sludge was freeze dried, sterilized by heating at 103 ^{o}C and stored at 4 ^{o}C until use. To determine K_d values of the investigated compounds, batch experiments were conducted for a range of initial concentrations of each compound (10, 40, 80, 150, 300 and 500 μg L^{-1}) to 3 g L^{-1} sludge and 100 mL tap water. Flasks were covered in order to inhibit photodegradation, agitated at 120 rpm on a shaking plate and samples were taken at the end of the experiment (24 h) for analysis of the target compounds in the water phase. All the experiments were performed at 22.0 ± 1.0 ^{o}C, while pH was 7.3 ± 0.2. Sorption experiments are described in **Paper A**.

Biodegradation experiments were conducted under batch conditions with AS in order to calculate biodegradation constants for each compound. These experiments were conducted in triplicates in order to determine the influence of aerobic/anoxic conditions, SRT and supplementary organic substrate on biodegradation kinetics. As described in **Paper A**, AS from a nitrifying municipal STP (Mytilene, Greece) was used for most biodegradation experiments. After being collected, biomass was left to settle and the supernatant was rejected and replaced with tap water. Afterwards, sludge was aerated for 48 hours and appropriately diluted to achieve the desired concentration. The experimental conditions used in different biodegradation batch experiments (A to G) are presented in Table 5.

Experiments were conducted in stoppered glass bottles that were constantly agitated on a shaking plate. The working volume in each reactor was 1 L and the mixed liquor suspended solids (MLSS) concentration was 3000 ± 200 mg L^{-1}. The target compounds were spiked using methanol solutions to obtain an initial concentration of around 30 μg L^{-1} for each microcontaminant in the reactors. To quantify biodegradation of micropollutants, homogenized samples of mixed liquor (10 mL) were collected after 0, 8, 24, 36, 48 and 72 hours. The concentrations of target compounds were determined in the dissolved and particulate phase using the analytical methods described below. In aerobic experiments, DO concentrations higher than 4 mg L^{-1} were achieved by using aeration through porous ceramic diffusers. In anoxic experiments, the reactors were initially purged with N_2 gas and a solution of sodium nitrate ($NaNO_3$) was added to provide an initial concentration of NO_3-N equal to 40 mg L^{-1}. To investigate the role of easily degradable substrate on target compounds biodegradation, synthetic

wastewater containing peptone, urea, yeast extract, and other micronutrients (Lozada et al., 2004) was added every 24 hours in order to achieve COD concentration equal to 200 mg L^{-1} in the appropriate flasks. To investigate the role of SRT on target compounds removal, aerobic experiments were also performed using biomass originating from a nitrifying STP that operated at SRT of 8 days (Athens, Greece). Finally, to investigate the effect of abiotic conditions on target compounds removal, batch experiments were performed with sodium azide (NaN_3, 0.2% w/v) to inactivate microorganisms activity. Most experiments (Experiments A to E) were conducted in triplicate; whereas experiments F to G were conducted without replication (Table 5). In all experiments the temperature was 22.0 ± 0.5 ^oC, while pH ranged between 7.2 and 8.2.

Table 5: Experimental protocol used in biodegradation batch experiments (initial concentration of target compounds: 30 μg L^{-1}; concentration of mixed liquor suspended solids, MLSS: 3000 ± 200 mg L^{-1}; experiments A to E: 3 replicates, experiments F to G: 1 replicate), from **paper A**.

Batch experiments	Constituents	Conditions	Sludge origin
A	Sludge + Target Compounds	Aerobic	STP A[1]
B	Sludge + Target Compounds	Anoxic	STP A
C	Sludge + Target Compounds + Organic Substrate	Aerobic	STP A
D	Sludge + Target Compounds + Organic Substrate	Anoxic	STP A
E	Sludge + Target Compounds	Aerobic	STP B[2]
F	Sterilized Sludge + Target Compounds + Organic Substrate + NaN_3	Aerobic	STP A
G	Sterilized Sludge + Target Compounds + Organic Substrate + NaN_3	Anoxic	STP A

[1]STP A (Mytilene) operated at SRT of 18 days; [2]STP B (Athens) operated at SRT of 8 days

2.1.3. Experiments with Continuous-Flow AS, MBBR and HMBBR systems

Three continuous flow systems were operated and compared regarding micropollutants removal. All of them were fully aerated lab scale systems that were fed constantly with raw sewage collected from the University Campus STP. All systems are briefly described below and further information can be found in **Paper B** and **Paper C**.

2.1.3.1. Systems Description and Operation

Three small scale continuous flow systems (Figure 9) were installed and operated in the laboratory under constant room temperature controlled by central air-conditioning system, at different time periods. In all bioreactors, the conservation of aerobic conditions and the adequate mixing of suspended and attached biomass were achieved by providing constant air supply, while DO concentration was higher than 4 mg L^{-1}.

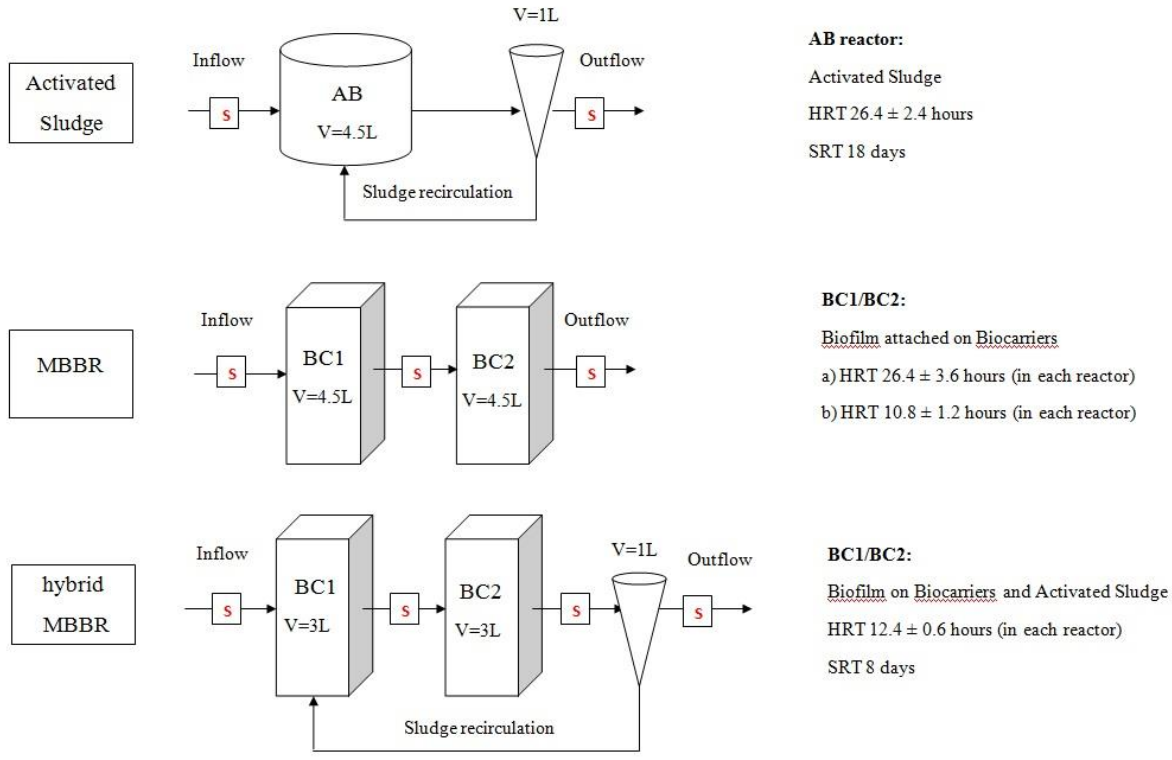


Figure 9: Schematic description of the continuous-flow biological treatment systems used in this study (sampling points are presented with an S).

The AS system consisted of an aerobic bioreactor (AB), with a working volume of 4.5 L, and a settling tank with a working volume of 1 L, from which sludge was recirculating to the bioreactor (Solid Retention Time, SRT: 18 d; HRT: 26.4 ± 2.4 h; organic loading: 0.25 ± 0.16 kg m^{-3} d^{-1}). The AS for AB start-up was taken from a nitrifying municipal STP (Mytilene, Greece), in summer 2014.

The MBBR system consisted of two aerobic bioreactors (BC1 and BC2) connected in series, with a working volume of 4.5 L each. Each bioreactor contained biocarriers (type K3, AnoxKaldnes™) at a filling ratio of 30%. The biocarriers were moving due to aeration in all parts of the reactor. The MBBR system was operated at two HRTs, in two different experimental cycles during summer and autumn 2014. A HRT of 26.4 ± 3.6 h (for each reactor) was applied in the first experimental cycle, providing a low substrate organic loading (MBBR-low), equal to 0.25 ± 0.16 kg m^{-3} d^{-1} for BC1 and 0.05 ± 0.03 kg m^{-3} d^{-1} for BC2. A lower HRT of 10.8 ± 1.2 h (for each reactor) was applied in the second experimental cycle in order to provide higher substrate organic loading (MBBR-high), equal to 0.60 ± 0.40 kg m^{-3} d^{-1} for BC1 and 0.17 ± 0.11 kg m^{-3} d^{-1} for BC2.

The HMBBR system consisted of two aerobic bioreactors (BC1 and BC2) connected in series, with a working volume of 3 L each. A settling tank, with a volume of 1 L, followed the two reactors, from which AS was recirculated to BC1. Each bioreactor contained both biocarriers (type K3, AnoxKaldnes, at a filling ratio of 30%) and AS. The AS was collected from a nitrifying municipal STP (Mytilene, Greece), while the biocarriers were taken from the laboratory scale MBBR system that has been previously operated. A HRT of 12.4 ± 0.6 h (for each reactor) was applied, providing a substrate organic loading equal to 0.64 ± 0.39 kg m^{-3} d^{-1} for BC1 and 0.11 ± 0.09 kg m^{-3} d^{-1} for BC2. The SRT of AS in the system was kept at 8 d.

2.1.3.2. Experiment with Micropollutants

All systems were operated for an appropriate time period in order to achieve stable performance and efficient removal of conventional pollutants. After this time period, the target compounds were spiked using methanol solutions to obtain a daily stable concentration inflow of approximately 20 μg L^{-1} of each investigated chemical. To evaluate the removal of target compounds in different systems and bioreactors, samples were taken during at least one week from different sampling points of each system (Figure 9). In these experiments target compounds were analyzed only in the dissolved phase,

as the study that came first proved that the compounds sorption on sludge was of minor importance (**Paper A**).

During the operation of continuous flow treatment systems, additional batch experiments were conducted using biomass from these systems. This was done in order to determine the biodegradation capacity of developed biomass (suspended or attached) and to obtain biodegradation kinetics for modelling purposes. In these experimental cycles, batch experiments were conducted in one replicate and are described in detail in **Paper B** and **Paper C**.

2.1.4. Batch Experiments for By-Products Identification

To identify the biotransformation products of target compounds in the HMBBR system, aerated batch experiments were conducted using biomass from the first bioreactor (BC1) where the greatest part of biodegradation was observed during the continuous flow experiment. Mixture of AS and biocarriers from BC1 was transferred to seven different glass bottles at a final volume of 200 mL. Each target compound was spiked in a different bottle at an initial concentration of 10 mg L^{-1}, while a control flask was also prepared containing biomass and methanol at an amount equal to that added in other reactors. All bottles were covered with aluminium foil and constantly agitated on a shaking plate. The total duration of the experiment was 24 h. Three homogenized samples (10 mL each) were taken from each reactor at 0, 6 and 24 h.

2.2. Analytical Methods

The analysis of parent compounds and the chemical analyses described in Paragraphs 2.2.1 and 2.2.3 were conducted in Water and Air Quality Laboratory of the Department of Environment, University of the Aegean. On the other hand, the analysis for by-products described in Paragraph 2.2.2 was conducted in the Laboratory of Analytical Chemistry of the Department of Chemistry, National and Kapodistrian University of Athens.

2.2.1. BTRs and BTHs analysis

For the investigation of target compounds fate, samples were filtered through pre-ashed glass fiber filters (GF-3 Macherey Nagel). Filtrates were collected, acidified to pH 3.0 ± 0.1 and stored at 4 ^{o}C until analysis. Filters were oven dried at 60 ^{o}C until constant weight and stored at -18 ^{o}C. Analysis of target

compounds in the dissolved and particulate phase was based on previously developed methods by Asimakopoulos et al. (2013b) and included Solid Phase Extraction (SPE) for liquid samples and sonication, followed by SPE clean-up step, for solid samples (Figure 10).

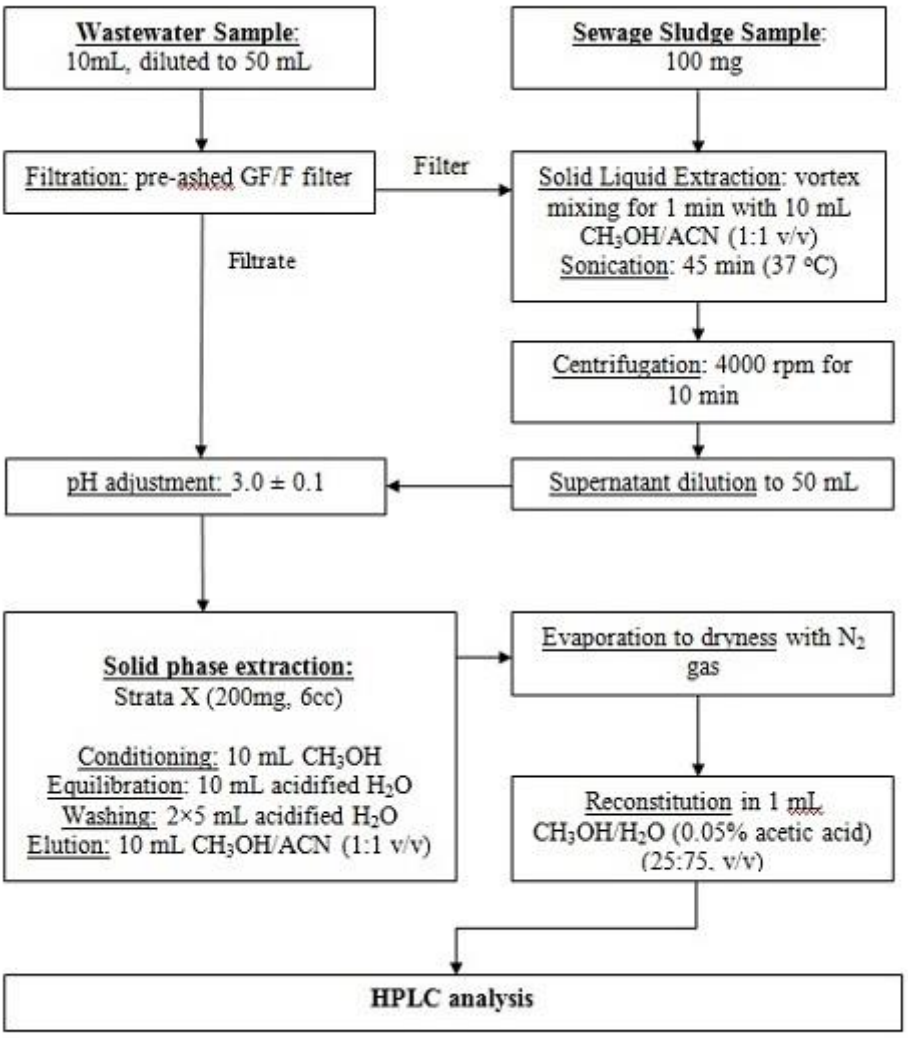


Figure 10: Schematic description of the applied analytical method for the determination of the target Benzotriazoles (BTRs) and hydroxy-benzothiazole (OHBTH) in wastewater and sludge samples.

Chromatographic analysis was performed by a Shimatzu (Japan) LC20-AD prominence liquid chromatographer associated with a SPD-M20A diode array detector (DAD) and a SIL-20AC auto sampler. The column was a Zorbax SB-C18 4.6 mm x 150 mm (5 μm) connected with a Zorbax SB-C18 pre-column (Agilent, USA). The column and pre-column were heated at 35 °C with a CTO-20AC column oven (Shimatzu-Japan). The mobile phase consisted of MilliQ grade water 0.05% acetic acid

(solvent A) and Acetonitrile (ACN; solvent B). Gradient elution was performed as follows: from 25% ACN to 75% ACN in 15 min, hold for 9 min and then decrease to 25% ACN in one minute. The system was equilibrated for 10 min with 25% ACN before each run. The total duration of the separation program was 35 minutes and the flow rate was 0.5 mL min^{-1}. The DAD was set at measurement wavelengths ranging from 190 to 300 nm, while all compounds were quantified using the signal at 254 nm. The identification of the six compounds in the sample was accomplished on the basis of their retention times and comparing their Ultraviolet (UV) spectrum in the standard solutions and in the samples. A typical chromatograph is presented in Figure 11.

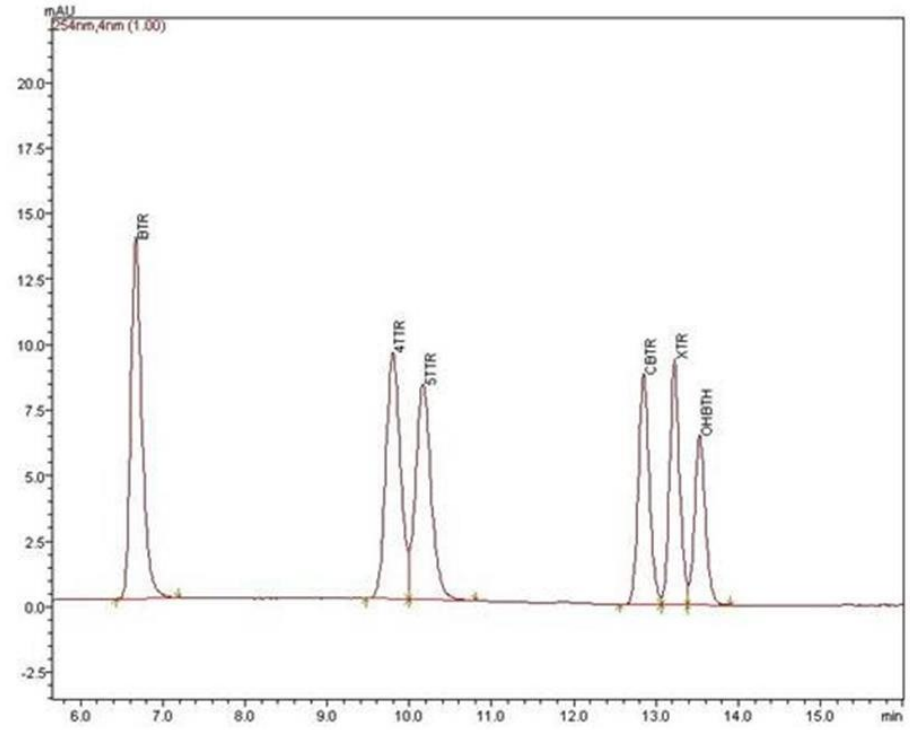


Figure 11: Chromatogram of the target compounds separation during HPLC analysis (methanolic standard solution containing 100 μg L^{-1} of each compound).

Validation of the analytical methods included analytical methods calibration, determination of limits of detection (LODs), assessment of precision and evaluation of trueness for both dissolved and particulate phase samples (Table 6). Analytical methods calibration was carried out for concentrations ranging from 10 to 500 μg L^{-1} and the response of the diode array detector was linear for all target compounds (R^2 > 0.99). Satisfactory recoveries and precision of the analytical procedures were achieved. For dissolved samples, the obtained LODs ranged from 17 (BTR) to 125 (CBTR) ng L^{-1};

whereas for particulate samples the LODs varied between 40 (BTR) and 555 ng g^{-1} dry sludge (5TTR). All relevant information presented in this paragraph can be retrieved from **Paper A**.

Table 6: Precision, trueness and limits of detection (LODs) and quantification (LOQs) of the analytical methods

Compound	Intra-day precision (RSD %, n = 6)	Inter-day precision (RSD %, n = 3)	Trueness (Recovery %, n = 4)	LOD (ng L^{-1} in sample)	LOQ (ng L^{-1} in sample)
Dissolved phase					
BTR	8.9	1.1	73.9 - 82.9	17	52
4TTR	12.0	9.6	36.1 - 54.8	28	84
5TTR	7.7	7.4	60.6 - 68.8	23	69
CBTR	9.3	6.7	72.7 - 82.0	125	376
XTR	9.7	10.1	60.1 - 85.0	107	322
OHBTH	10.4	10.9	69.8 – 73.0	30	90
Compound	**Intra-day precision (RSD %, n = 6)**	**Inter-day precision (RSD %, n = 3)**	**Trueness (Recovery %, n = 4)**	**LOD (ng g^{-1} in sample)**	**LOQ (ng g^{-1} in sample)**
Particulate phase					
BTR	10.8	7.4	59.8 - 60.8	40	118
4TTR	10.5	6.6	53.6 - 77.5	368	1104
5TTR	11.0	11.6	67.1 - 73.5	555	1666
CBTR	13.8	14.0	64.8 - 69.1	132	397
XTR	11.1	11.3	50.8 - 54.0	236	709
OHBTH	6.5	5.9	70.1 - 74.8	72	216

2.2.2. Analysis of BTRs and BTHs By-Products

For the investigation of transformation products, samples were initially filtered through glass fibre filters (GF-3 Macherey Nagel),1.5 mL of each sample was filtered through 0.2 μm RC filter and collected. Filtrates were stored at -18oC until analysis. A LC-HR-MS/MS analysis Ultrahigh-performance liquid chromatography (UHPLC) system (DionexUltiMate 3000 RSLC, ThermoFisherScientific, Germany), coupled with a quadrupole-time-of-flight high-resolution mass

spectrometer (UHPLC-QToF-MS) (Maxis Impact QTOF, Bruker, Bremen, Germany) was used for transformation products identification. The chromatographic separation was performed using a Thermo Acclaim RSLC C18, 2.2μm 120 Å, 2.1x100 mm column. The gradient program for both positive and negative mode is presented in Table S5. Methanol (solvent A) and water:methanol (90:10) (solvent B) both amended with 0.01% formic acid and 5 mM ammonium formate was used as mobile phase for positive ionization and methanol and water:methanol (90:10) both amended with 5 mM ammonium acetate as an eluent for negative ionization mode. A sodium formate solution (10 mM) was always introduced between 0.1 to 0.3 min in the beginning of every chromatographic run through direct infusion at a flow rate of 50 μL h-1 to compensate for mass drifts and for internal mass calibration. Sodium formate solution was also used to perform daily external calibration in QTOFMS. The sodium formate calibration mixture consists of 10 mM sodium formate in a mixture of water/isopropanol (1:1).

The QToF mass spectrometer was equipped with an electrospray ionization interface (ESI) operating both in positive and negative ionization mode. Operation parameters were: capillary voltage, 2500 V; end plateoffset, 500 V; nebulizer pressure, 2 bar (N2); drying gas, 8 L min−1(N2); and drying temperature, 200 °C.Data were acquired through broad-band collision induced dissociation (bbCID) mode, providing MS and MS/MS spectra simultaneously under positive and negative electrospray ionization (two separate runs). HR-MS data was recorded within a mass-to-charge (m/z) range of 50–1000 for each sample, at 2 Hz spectra rate and at a continuously alternatively collision energy of 4 eV (low energy, LE) and 25 eV (high energy, HE) in the collision cell Q2, for full-scan and MS/MS data, respectively. For masses corresponding to plausible transformation products (TPs), the fragmentation performed in Auto MS/MS mode with an inclusion list. For masses corresponding to the detected plausible transformation products (TPs), MS/MS spectra was subsequently acquired with data dependent acquisition in Auto MS/MS mode with an inclusion list.

For TPs' identification, the samples were screened for the exact masses of potential TPs according to a suspect database that was compiled by the online pathway prediction system hosted by EAWAG institute (EAWAG-PPS) without the "relative reasoning mode". Two generations of TPs for each BTR and OH-BTH were predicted. MetabolitePredict (Bruker, Bremen, Germany),was also used for the prediction of possible phase I & II metabolites as well as cytochrome P450 metabolites, to extend the possible candidates for screening (Bletsou et al., 2015). For instance, monohydroxylation of benzotriazoles is not predicted by EAWAG-PPS, but it is predicted by MetabolitePredict software.

Finally, already known and reported metabolites from the literature were added to the database (Liu et al., 2011; Huntscha et al., 2014).

A data-processing software (TargetAnalysis 1.3, Bruker) was used for the suspect screening of plausible transformation products. All the time interval samples were screened, in both positive and negative ionization modes, for the determination of suspect TPs from the database. The characterization of an exact mass as a possible TP was based on the following criteria, deltaRT ≤ 0.10 min, mass error ≤ 5 ppm, isotopic fit: ≤ 1000 mSigma, intensity threshold >500 (+ESI) and >200 (-ESI) as well as, absence from the blank samples and occurrence of a time trend (Li et al., 2013). The potential TPs were subjected to MS/MS experiments via AutoMSmode with an inclusion list in order to obtain the MS/MS spectra and the fragments for further assignment of molecular formulas and structure elucidation. The SmartFormula algorithm was used to apply the sum formulae of the protonated or deprotonated ion and fragments (mass error and isotopic fit was also calculated). SmartFormula uses element restrictions for C, H, N and O, [M±H]±for positive and negative ion mode, mass tolerance of 5 ppm, the hydrogen to carbon ratio (H/C) ranges from 0 to 3, it checks for ring and double bonds and allows even electron configuration for the MS peaks and both odd and even electron configuration for MS/MS peaks.

2.2.3. Analysis of other Chemical Parameters

Analysis of COD, NH_4-N, NO_3-N, Total Suspended Solids (TSS) and MLSS were performed according to Standard Methods (APHA, 1998), T, DO and pH were measured using portable instruments. Biofilm solids were determined by the difference in weight of dried carriers (105 °C for ≥1 h) before and after removal of biofilm. Removal of biofilm solids were made in H_2SO_4 solution (4 M) through mechanical shaking and ultrasonication, followed by thorough brushing, as described by Falås et al. (2012). Stereoscopic and microscopic observations were conducted periodically in order to have an overview of biofilm development and AS characteristics (all this information is available in **Paper B**).

2.2.4. Calculations and Equations

Various equations were used for treatment of experimental results and calculation of different constants. The main calculations are described, while detail information on equations can be found in **Papers A, B, C**.

Sludge-water distribution coefficients, K_d values, (L g^{-1}) of target compounds were estimated from batch sorption experiments using Equation 1:

$$K_d = \frac{q_e}{C_e} \quad (1)$$

Where, q_e is the concentration of target compound in the particulate phase (μg g^{-1}) and C_e is the concentration of target compound in the dissolved phase (μg L^{-1}).

The half-lives of target compounds in aerobic and anoxic biodegradation experiments were estimated using first-order kinetics, (Equations 2, 3):

$$C_t = C_o e^{-kt} \quad (2)$$

$$t_{1/2} = \frac{\ln 2}{k} \quad (3)$$

Where C_t and C_0 are the total (dissolved + particulate) target compound concentrations in batch experiment at time t and $t = 0$, respectively, (μg L^{-1}), k is the biodegradation coefficient (d^{-1}) and $t_{1/2}$ is the half-life (d).

Pseudo first-order biodegradation rate coefficient, k_{bio}, normalized to mixed liquor suspended solids (L g_{MLSS}^{-1} d^{-1}) was calculated for each biodegradation experiment using Equation 4, (Ziels et al., 2014).

$$\ln \frac{C_t}{C_0} = -k_{bio}\left(\frac{MLSS}{1 + K_d MLSS}\right) \times t \quad (4)$$

In order to predict the removal and fate of target compounds during activated sludge process, Equations 5 and 6 were used (Tchobanoglous et al., 2002) for the two full-scale STPs operating at SRTs of 18 d and 8 d (Figure 8, **Paper A**):

$$M_{in} = M_{bio-anox} + M_{bio-aer} + M_{sorbed} + M_{out} \quad (5)$$

Where M_{in} and M_{out} are the masses of target compounds in raw and treated wastewater, respectively (mg d^{-1}), $M_{bio-anox}$ and $M_{bio-aer}$ are the masses of target compounds that are biodegraded in the anoxic and the aerobic bioreactor, respectively (mg d^{-1}) and M_{sorbed} the mass of each target compound removed with excess sludge from the bioreactors (mg d^{-1}).

$$C_{in}Q_{in} = (k_{bio-anox}C_{out}XV_{anox}) + (k_{bio-aer}C_{out}XV_{aer}) + \left(\frac{XVK_dC_{out}}{SRT}\right) + (Q_{out}C_{out}) \quad (6)$$

Where C_{in} and C_{out} are the concentrations of target compounds in raw and treated wastewater, respectively (mg m^{-3}), Q_{in} and Q_{out} are the flow rates in raw and treated wastewater, respectively (m^3 d^{-1}), $k_{bio-anox}$ and $k_{bio-aer}$ are the experimentally calculated normalized biodegradation constants under anoxic and aerobic conditions, respectively (L g_{MLSS}^{-1} d^{-1}), X is the concentration of MLSS in full-scale bioreactors (g_{MLSS} L^{-1}), V_{anox} and V_{aer} are the volumes of anoxic and aerobic full-scale bioreactors, respectively (m^3), K_d is the experimentally calculated sludge-water distribution coefficient (L g^{-1}) and SRT is the sludge residence time in activated sludge bioreactors (d).

Micropollutants removal in laboratory scale reactors (**Paper B and C**) was calculated according to Equation 7.

$$\text{Re}\,moval = \left(1 - \frac{C_{out}}{C_{in}}\right) \times 100 \quad (7)$$

Where C_{in} is the concentration of target compound in raw wastewater (μg L^{-1}) and C_{out} the concentration in treated wastewater of each examined reactor (μg L^{-1}).

Specific removal rate for each compound and type of biomass was calculated according to Equation 8.

$$Specific \cdot \text{Re}\,moval \cdot Rate = \left(\frac{C_{in}Q_{in} - C_{out}Q_{out}}{XV}\right) \quad (8)$$

Where Q_{in} and Q_{out} are the flow rates of raw and treated sewage, respectively (L d^{-1}), X is the concentration of attached or suspended biomass (g L^{-1}) and V is the volume of each bioreactor (L).

Predicted removal in AS and MBBR continuous-flow systems was estimated using Equation 9 (**Paper B**).

$$\text{Pr}\,edicted \cdot \text{Re}\,moval = 1 - \left(\frac{1}{(1+k_1\tau_1)(1+k_2\tau_2)}\right) \quad (9)$$

Where τ is the hydraulic retention time for each reactor; in the case of the MBBR system (τ_1, τ_2), while for the AS system only one reactor was used (τ_1) and k is the first-order biodegradation rate constant calculated in batch experiments (**Paper B**).

Equations 5 and 6 with the appropriate variations were used for the prediction of target compounds removal in the HMBBR system, as described in **Paper C**.

2.2.5. Statistical Analysis

For data evaluation, the software GraphPad Prism 5 for Windows was used. Furthermore the same software was used in order to conduct appropriate statistical analysis, when needed. In order to compare the removal values and specific removal rates in continuous flow systems, one-way ANOVA was used with Tukey-Kramers post-test for significant differences between groups. T-bars in figures presented in the results represent 95% confidence interval, while the letters and symbols indicate statistical differences at 95% confidence level.

2.2.6. Chemicals and Reagents

Analytical standards of XTR and CBTR were supplied by Sigma-Aldrich (USA). BTR was purchased by Merck (Germany), 4TTR by Fluka (Switzerland), 5TTR by Acros Organics (Belgium) and OHBTH by Alfa Aesar (USA). Stock solutions of individual compounds were prepared in Methanol (MeOH) at 1000 mg L^{-1} and kept at −18 ^{o}C. MeOH (HPLC-MS grade) and ACN (HPLC grade) were purchased by Merck (Germany) and Fisher (USA), respectively. The SPE cartridges used for samples' clean-up were Strata-X (33u Polymeric Reversed Phase, 200mg/6ml) and they were supplied by Phenomenex (USA). HPLC grade water was prepared in the laboratory using a MilliQ/MilliRO Millipore system (USA). Ultra-pure HCl (32%) was purchased by Merck (Germany).

3. Results and Discussion

3.1. Investigation of BTRs and OHBTH sorption and biodegradation onto Activated Sludge

As a first step of this PhD thesis (**Paper A**), the sorption of target compounds onto AS and their biodegradation potential by AS was examined. Several sets of batch experiments were conducted and the obtained experimental values were used for the determination of target compounds fate in two large scale STPs.

The sorption of target compounds was studied in parallel batch experiments, containing different concentrations of the investigated compounds. The applied experimental protocol has been described in Paragraph 2.1.2. The obtained sorption constants are presented in Table 7, proving the general hypothesis that the compounds have a weak tendency to sorb onto organic matter. These results are presented in detail in **Paper A** and are in accordance with a previous study that calculated sorption constants of BTR and OHBTH using full-scale monitoring data of a Greek STP (Stasinakis et al., 2013).

Table 7: Sludge-water distribution coefficients (K_d) determined in batch experiments with AS. The 95% confidence intervals of the measured K_d values are given in parenthesis.

Compound	K_d (L Kg^{-1})	R^2
BTR	220 (± 9)	0.993
4TTR	170 (± 48)	0.870
5TTR	165 (± 14)	0.979
CBTR	242 (± 5)	0.998
XTR	87 (± 17)	0.930
OHBTH	147 (± 29)	0.893

As described above, different batch experiments were also conducted using activated and sterilized sludge to study the biodegradation potential of BTRs and OHBTH during wastewater treatment in a typical STP. Furthermore, the role of different factors on biodegradation kinetics was investigated.

Monitoring of the total (dissolved + particulate) concentrations of target compounds in abiotic experiments showed no removal of these compounds due to abiotic causes. The experiment on micropollutants' partitioning during biodegradation experiments showed, as expected, that the greatest part of the target compounds were in the dissolved phase and this part significantly decreased during the experiment. In all biotic experiments, no significant removal of 4TTR was noticed; whereas removal of 5TTR ranged between 20 and 38%, therefore no biodegradation constants were calculated for these two compounds. Previous studies have also reported no removal of 4TTR during AS process (Weiss et al., 2006, Herzog et al., 2014a), while biodegradation of 5TTR seems to be slow (complete removal after 91 days, according to Liu et al., 2011) which is enhanced by adaptation of microorganisms (Herzog et al., 2014b). For the four other compounds (BTR, CBTR, XTR and OHBTH), which were removed to an extent higher than 50% during biodegradation experiments, a first order kinetic equation was fitted taking into concern the three individual experiments conducted for each condition examined. The first order biodegradation rate constant (k) and the half life of each compound were calculated with 95% confidence intervals. Furthermore the biodegradation rate constant was normalized to the amount of biomass (k_{bio}) in order to compare values that occurred from each experiment. The calculated values of biodegradation constants are presented in Table 8.

Table 8: First order kinetics (k), half-life values ($t_{1/2}$) and biodegradation constants (k_{bio}) calculated in batch experiments with AS, taken from a municipal STP, under different experimental conditions. The 95% confidence intervals of measured values are given in parenthesis.

Compound	Experiment	k (d^{-1})	Half life (h)	R^2	k_{bio} (L g_{SS}^{-1} d^{-1})
BTR	Aerobic, SRT 18d	0.38±0.13	44±18	0.735	0.22±0.08
	Anoxic, STR 18 d	0.41±0.12	40±12	0.807	0.24±0.07
	Aerobic with substrate, STR 18 d	0.73±0.12	23±4	0.947	0.41± 0.07
	Anoxic with substrate, SRT 18 d	0.59±0.12	29±6	0.914	0.33± 0.07
	Aerobic, SRT 8 d	0.37±0.14	45±21	0.810	0.21±0.08

Compound	Experiment	k (d^{-1})	Half life (h)	R^2	k_{bio} (L g_{SS}^{-1} d^{-1})
CBTR	Aerobic, SRT 18d	0.54±0.06	31±3.5	0.984	0.33±0.04
	Anoxic, STR 18 d	0.75±0.18	22±5.7	0.886	0.45±0.11
	Aerobic with substrate, STR 18 d	0.83±0.24	20±6.4	0.855	0.49±0.14
	Anoxic with substrate, SRT 18 d	0.90±0.25	18±5.5	0.869	0.54±0.15
	Aerobic, SRT 8 d	0.36±0.06	47±17	0.972	0.21±0.04
XTR	Aerobic, SRT 18d	0.86±0.35	20±9.5	0.759	0.39±0.16
	Anoxic, STR 18 d	0.88±0.26	19±6.0	0.865	0.40±0.12
	Aerobic with substrate, STR 18 d	1.19± 0.54	14±8.0	0.759	0.52±0.24
	Anoxic with substrate, SRT 18 d	0.79±0.29	21±8.8	0.801	0.35±0.13
	Aerobic, SRT 8 d	0.64±0.30	26±16	0.790	0.29±0.14
OHBTH	Aerobic, SRT 18d	0.77±0.34	22±12	0.712	0.40±0.17
	Anoxic, STR 18 d	1.23±0.43	14±5.5	0.849	0.63±0.22
	Aerobic with substrate, STR 18 d	2.58±0.72	6.5±1.9	0.937	1.29±0.36
	Anoxic with substrate, SRT 18 d	1.48±0.33	11±2.6	0.943	0.74±0.16

Compound	Experiment	k (d^{-1})	Half life (h)	R^2	k_{bio} (L g_{SS}^{-1} d^{-1})
	Aerobic, SRT 8 d	0.71±0.34	24±15	0.783	0.36±0.18

According to these results, the lowest half-life value ($t_{1/2}$ = 6.5 h) was calculated for OHBTH under aerobic conditions, SRT of 18 d and in the presence of supplementary organic substrate. Concerning the effect of SRT on biodegradation kinetics of the investigated compounds, except for CBTR, no effect was observed for the other micropollutants. This observation indicate that microorganisms capable of degrading these compounds are present in both AS systems (Athens and Mytilene STPs), operating at SRT of 8 and 18 days and as a result biodegradation of these compounds can be expected in all nitrifying conventional and extended aeration AS systems.

Experiments with supplementary organic substrate showed no competitive substrate inhibition or catabolic repression of target compounds biodegradation in the presence of easily degradable organic substrate. On the contrary, the addition of organic substrate resulted in decreased half-life values of BTR (under aerobic and anoxic conditions), CBTR (under aerobic conditions) and OHBTH (under aerobic conditions). Having in mind that a) the low concentrations of micropollutants added in these experiments (μg L^{-1}) cannot support a significant growth of specified degrading bacteria and b) no lag phase was noticed in degradation experiments; it therefore seems that biodegradation of target compounds occurs due to co-metabolic phenomena by microorganisms utilizing a wide range of carbon sources. The aerobic co-metabolic biotransformation of BTR due to hydroxylation of the aromatic benzene ring and methylation of the triazole ring was recently shown by Huntscha et al. (2014). Further information concerning these experiments can be found in **Paper A**.

The calculated sorption and biodegradation constants were used to predict the contribution of different mechanisms on BTR, CBTR, XTR and OHBTH removal duringactivated sludge process. For this reason, Equation 6 was applied for two STPs, operating at SRT of 8 and 18 days. According to the model's estimations, all target compounds are partially removed during the activated sludge process, while slightly higher removal efficiency is expected to occur in the STP operating at higher SRT, ranging from 29% for BTR to 46% for OHBTH (Table 9). The partial removal of BTR, CBTR, XTR and OHBTH has also been reported in monitoring studies of full-scale STPs (Weiss et al., 2006, Liu et al.,

2012, Stasinakis et al. 2013). Due to the low K_d constants of the investigated compounds, the contribution of sorption was of minor importance for their removal and varied from 0.5% (XTR, SRT: 18 d) to 2.7% (CBTR, STR: 8 d) (Table 9).

Table 9: Contribution of different mechanisms to the removal of the investigated compounds during activated sludge treatment in typical STPs operating either at Solid Residence Time (SRT) of 8 d and 18 d. Predictions are based on the experimentally determined sorption and biodegradation constants.

Compound	Predicted Removal (%) in a STP operating at a SRT of 18 d			
	Anoxic biodegradation	Aerobic biodegradation	Sorption	Total
BTR	9.7	18	1.5	29
CBTR	16	22	1.4	39
XTR	14	26	0.5	41
OHBTH	20	25	0.8	46
Compound	**Predicted Removal (%) in a STP operating at a SRT of 8 d**			
	Anoxic biodegradation	**Aerobic biodegradation**	**Sorption**	**Total**
BTR	7.8	14	2.6	24
CBTR	14	13	2.7	30
XTR	12	17	1.0	30
OHBTH	17	19	1.5	38

Comparing different STPs, higher removal due to sorption was observed in the case of lower SRT and this is due to the higher production and removal of excess sludge under these conditions. On the other hand, biotransformation in aerobic and anoxic bioreactors seems to be the major mechanism for their removal, ranging from 22% (BTR, SRT: 8 d) to 45% (OHBTH, STR: 18 d) (Table 9). As aerobic biodegradation constants ($k_{bio\text{-}aer}$) were similar or smaller than those calculated under anoxic conditions ($k_{bio\text{-}anox}$) for all target compounds (Table 8); the higher contribution of aerobic bioreactor on their removal is mainly due to the greater volume of aerobicreactor and to the relative greater mass of involved microorganisms comparing to the anoxic.

To investigate model's sensitivity to different factors which could affect the prediction of the removal of the investigated compounds, three different scenarios were tested. Specifically, by increasing MLSS concentration in anoxic and aerobic bioreactors from 3000 mg L^{-1} to 5000 mg L^{-1}, an increase of the total removal efficiency equal to 10-13% was calculated for target compounds (Table S1). For the case that

k_{biol} constants were 20% higher than those experimentally calculated, 3% (BTR) to 5% (CBTR) higher removal would be observed in a STP operating at SRT of 18 d (Table S1). Similarly, an overestimation of k_{biol} by 20% would decrease total removal of these compounds from 4% (BTR) to 5% (OHBTH, CBTR, XTR). These results indicate that the operation of full-scale bioreactors at higher MLSS concentrations with constant hydraulic retention time could improve removal of these compounds, while possible errors on calculation of biodegradation constants affect slightly their predicted elimination rates.

3.2. Comparison of BTRs and OHBTH biodegradation in AS and MBBR systems

As a second step of this PhD thesis, lab-scale sewage treatment systems were tested for the biological removal of examined BTRs and OHBTH. Two different systems were operated in parallel in order to compare each systems performance concerning micropollutants elimination. Two types of biomass were actually compared, suspended (AS) and attached (MBBR). In the first experimental cycle, both AS and MBBR were operated under the same conditions concerning the HRT and organic loading. In the second cycle, the MBBR was operated at a lower HRT, which was closer to the typical operating parameters of an MBBR and lead to a higher organic loading of the system (MBBR-high). The percent removal was calculated and the capacity of each biomass to remove target compounds was examined by calculating the specific removal rate. Furthermore, batch biodegradation experiments were conducted in order to calculate and compare biodegradation constants for suspended and attached biomass. These constants were used in order to predict target compounds removal in differently loaded systems and were compared with measured removal. All these results have been presented in detail in **Paper B,** while the major findings can be found below.

Concerning the operation of the two systems (Table S2), both eliminated adequately organic loading from wastewater, achieving average dissolved COD removal equal to 86% (MBBR-low) and 90% (AS). Both systems were also able to remove NH_4-N sufficiently (average removal 93 - 95%). During microscopic observations, protozoa, rotifers and filamentous bacteria were identified in the AS system, indicating a stable and mature environment. Metazoa and protozoa were also observed in the MBBR system. In the AS system, the MLSS concentration was close to the typical in an STP (2230 ± 290 mg L^{-1}), while in the MBBR system, a thicker biofilm developed in the first bioreactor, BC1 resulting to a

higher concentration of biomass. Despite the thinner biofilm in BC2, the developed biomass had a greater ability to nitrify. Furthermore, in the high loaded MBBR system, a thicker biofilm was observed in both bioreactors. In the first experimental cycle (low loaded MBBR), on average 170 mg of NH_4-N were removed per day and per gram of biomass in BC1, while 250 mg d^{-1} g^{-1} were removed in BC2. A similar trend was also observed during the second experimental cycle (high loaded MBBR), with nitrification rates being even higher in both reactors (on average 295 mg d^{-1} g^{-1} in BC1 and 480 mg $d^{-1}g^{-1}$ in BC2). Furthermore, in the high loaded MBBR system, a thicker biofilm was observed in both bioreactors.

As proved by the results presented in **Paper A**, the compounds are not expected to be degraded due to abiotic mechanisms, and are poorly sorbed onto biomass. Therefore the observed removal of target compounds in each system was mainly due to biodegradation. Their average removal varied from 43 to 76% for BTR, 8 to 69% for 4TTR, 0 to 53% for 5TTR, 42 to 49% for CBTR, 9 to 43% for XTR and 80 to 97% for OHBTH (Figure 12), indicating that none of the compounds was totally eliminated during wastewater treatment. Except for CBTR that was removed at the same rate regardless of the treatment type, all other compounds were eliminated to a different degree, depending on the system used.

In order to compare the removal efficiency of a suspended-growth and an attached-growth system operating in parallel under the same organic loading conditions and HRT, AS system and BC1 of MBBR-low system were used. According to Figure 12, the removal of 4TTR, 5TTR and XTR was similar in both systems, whereas statistically significant differences were observed for BTR (higher in AS), CBTR (higher in AS) and OHBTH (higher in MBBR), indicating that the application of same organic loading and HRT does not necessarily lead to same removal for all micropollutants. The increase of HRT in the low loaded MBBR system via the addition of a second reactor (BC2) enhanced to some degree the removal of micropollutants (up to 15%) but complete removal was not achieved. Similarly to the current study, Ahmadi et al. (2015) observed a moderate increase of diethylphthalate and diallylphthalate removal when HRT was increased from 3 to 12 h in a MBBR laboratory scale system.

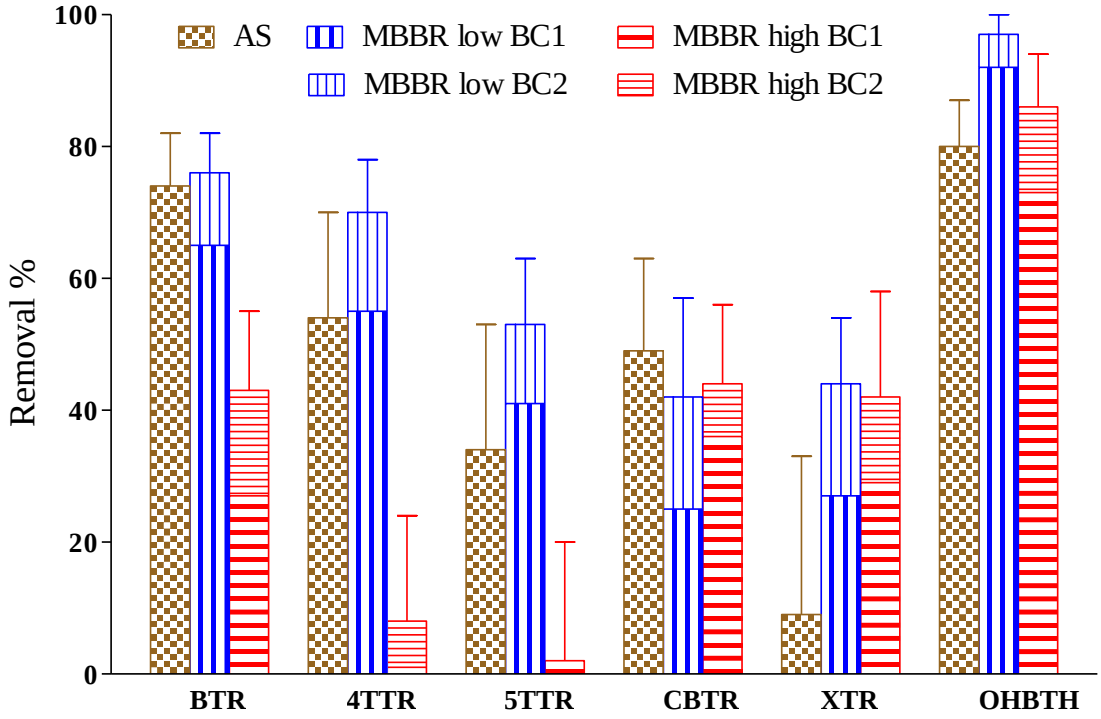


Figure 12: Removal (%) of target compounds in AS and MBBR system operated under low (MBBR-low) and high organic loading (MBBR-high) conditions (t-bars represent 95% confidence interval). The contribution of each bioreactor (BC1 and BC2) to target compounds removal is also shown.

When the MBBR system was operated under a higher organic loading (2nd experimental cycle), the total removal of XTR and CBTR was the same with low loaded MBBR. On the other hand, statistically lower removal was observed for OHBTH, BTR, 4TTR, while 5TTR was not eliminated at all (Figure 12). Beside the increased biomass developed in high loaded MBBR (Table S2), it seems that the increase of the organic loading in the MBBR system decreased its capacity to remove some of the target compounds. So far, limited results have been published in the literature for the role of organic loading on the removal of micropollutants. Ahmadi et al. (2015) using two phthalic acid esters as the sole carbon source reported that the increase of organic loading from 0.73 to 1.46 kg COD m^{-3} d^{-1} had not actual effect (<1%) on their removal in a MBBR, while no other studies are available in the literature for the range of organic loadings applied in the current study (0.25 to 0.60 kg m^{-3} d^{-1}) and for the added concentrations of micropollutants (μg L^{-1} levels).

As the biomass amount was not the same in all bioreactors (Table S2), the specific removal rate (as μg per g and day) was calculated for each micropollutant to compare the ability of biomass developed in

each system to remove the target compounds. According to the results presented in Figure 13 for total specific removal rate, the attached biomass developed in MBBR systems presented statistically significant higher ability to biodegrade all target compounds comparing to the suspended biomass of AS system.

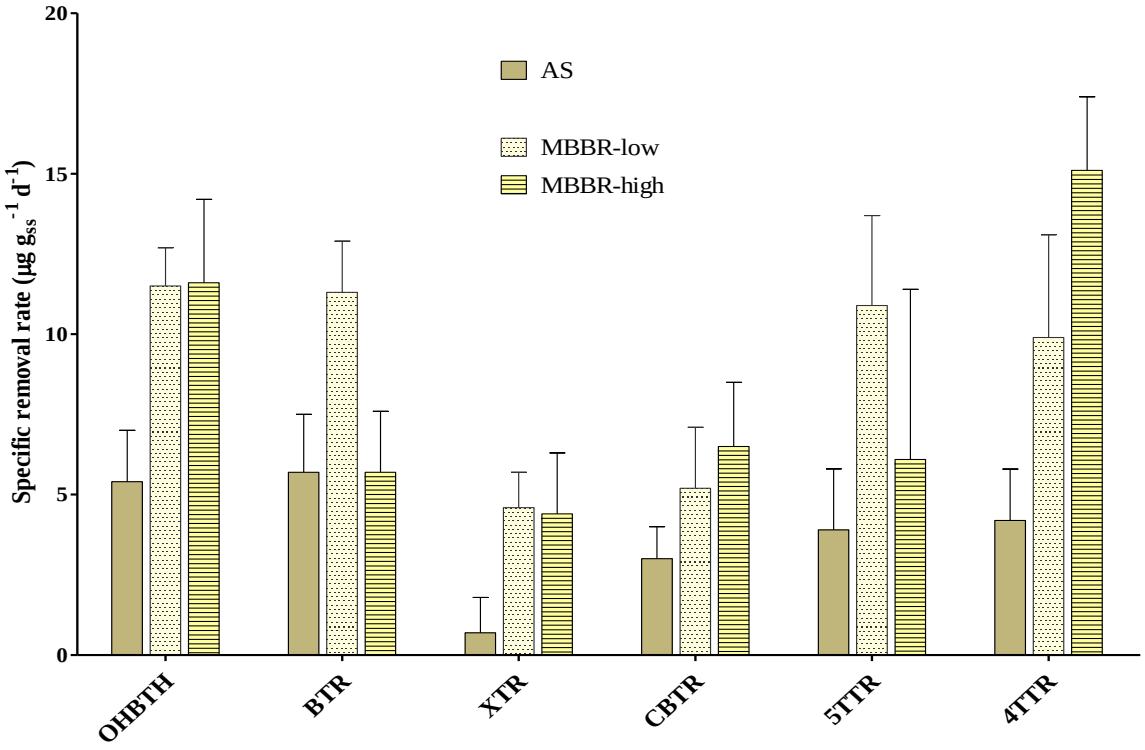


Figure 13: Mass of micropollutants removed per mass of biomass and day during continuous flow experiments with Activated Sludge (AS), Biocarriers under low loading conditions (MBBR-low) and Biocarriers under high loading conditions (MBBR-high) (values in bold indicate statistically significant differences).

In the low loaded MBBR system, these values ranged between 4.6 (XTR) to 11.3 μg g^{-1} d^{-1} (BTR), while similar (for OHBTH, XTR, CBTR) or lower values (for BTR and 5TTR) were calculated in high loaded MBBR system. This general advantage of the attached biomass over the suspended is probably due to the higher residence time of biomass onto carriers that could allow a richer biodiversity through the protection of slow growing bacteria from washout, which might be capable to remove micropollutants. In a recent study, Zhang et al. (2015) observed significant differences on the microbial communities established in suspended and attached biomass on phylum and genus level. Moreover, Edwards and

Kjellerup (2013) reported that a large variety of species of microorganisms is included in biofilms, whereas all of them contribute to each other's metabolic needs.

To investigate whether biomass with the same ability to remove our target compounds is grown in different bioreactors of the MBBR system, specific removal rates were also calculated for BC1 and BC2 of both MBBR systems (Figure S1). Differences were observed for each biomass and for each compound, indicating that biomass with different ability to remove micropollutants can be developed in each bioreactor of a MBBR system and BC2 seem to have a significant role in the development of microorganisms with higher capability to biodegrade micropollutants. It is known that the development of attached biomass is strongly affected by the wastewater characteristics (pH, temperature, type of bioavailable organic compounds, abundance of nutrients) and the operational conditions of the system (organic loading, aeration rate). The existence of low concentrations of micropollutants could also affect bacterial behaviour. In a recent study, it was reported that even small concentration of a xenobiotic compound (0.1 μg L^{-1} for PFOA and PFOS and 0.5 μg L^{-1} for triclosan) can provoke increase of extracellular polymers (EPS) in sludge, therefore affecting the transfer of substances from the mixed liquor to the interior of the flocs or the biofilm (Pasquini et al., 2013). This could decrease the amount of micropollutants available to microorganisms and therefore decrease their removal.

As in previous experiments, biodegradation constants (k and k_{bio}) were calculated by using first order equations and normalizing them to the amount of biomass. It should be mentioned that, 4TTR and 5TTR were not eliminated at high rates in batch experiments, therefore in some cases biodegradation constants could not be calculated. In most cases the biofilm (especially the biofilm developed in BC2) presented higher biodegradation constants over the AS. As can be seen in Table S3, the biofilm developed in the MBBR system (under high loading conditions) presented high constants for most compounds, with higher values observed when the organic loading in the beginning of the batch experiment was high. Among target compounds, the highest k_{bio} were obtained for CBTR, BTR and OHBTH and were 6.7, 5.6 and 4.8 L g_{SS}^{-1} d^{-1}, respectively. Regarding the role of COD on biodegradation kinetics, similarly to AS experiments, the increase of COD enhanced biodegradation of target compounds. These results indicate that co-metabolic phenomena are also responsible for the biodegradation of target compounds in attached biomass systems, as previously observed in **Paper A**.

To investigate how well biodegradation constants predict the removal of target compounds in continuous-flow systems, Equation 9 was used to predict the removal of each target compound and the predicted removal efficiencies are compared with measured removal efficiencies as shown in Figure 14.

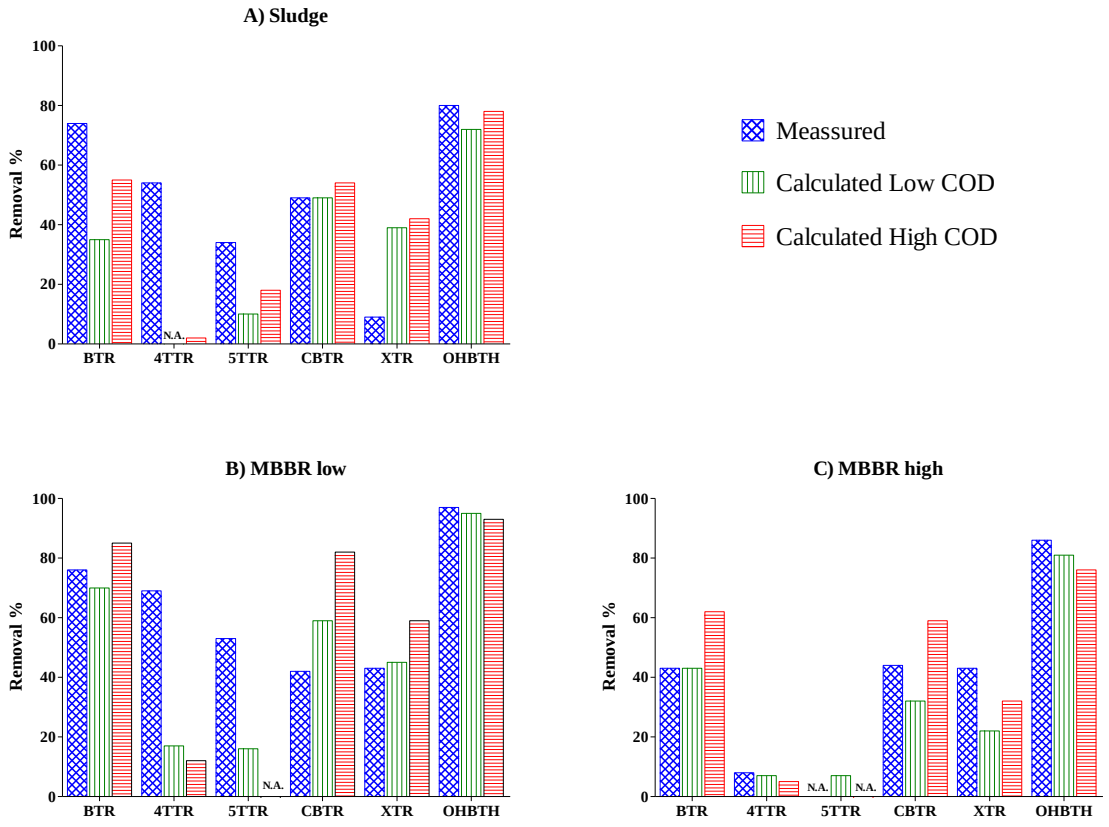


Figure 14: Measured and calculated removal in AS (A), MBBR-low (B) and MBBR-high system (C). Removal was calculated for low and high organic loading conditions.

The predicted removal by AS was very close to the observed removal for CBTR and OHBTH. For XTR, the measured removal was much lower than the predicted, while on the other hand BTR was actually removed at a higher extent (74%) than predicted (35% and 55%). Little removal was predicted for 4TTR and 5TTR which is quite different from that is observed in the continuous-flow system (Figure 14A). The differences might be due to the fact that the biomass used in batch experiments for the calculation of kinetics was not the same as that used in the continuous flow experiment. These observations indicate that for 4 out of 6 target compounds, care should be given on batch biodegradation kinetics

used for predicting their removal in full-scale systems, as the origin of biomass seem to affect the results.

Among MBBR systems, as it was expected, better prediction was achieved for MBBR-high as the biomass used for batch and continuous-flow experiments was the same. Specifically, the behaviour of BTR, 4TTR, 5TTR and OHBTH was predicted sufficiently, while minor fluctuations were observed for CBTR and XTR (Figure 14C). Regarding MBBR-low system, the use of Equation 4 predicted sufficiently 3 out of 6 (BTR, XTR, OHBTH). However, significant differences were observed especially for 4TTR and 5TTR (Figure 14B).

3.3. Study of BTRs and OHBTH fate and removal in HMBBR system

As a last step of this PhD Thesis, a HMBBR lab-scale system was used and the elimination of target micropollutants was compared with that observed in AS and MBBR systems. The removal of target compounds from sewage was investigated by monitoring the system and by conducting batch experiments. The calculated biodegradation constants were used to predicti the contribution of each type of biomass in elimination of target compounds. Additionally, possible biotransformation by-products were identified by conducting batch biodegradation experiments for each compound. All these results are presented in **Paper C**.

The HMBBR system was stable during the whole experimental period and achieved sufficient removal of dissolved COD (87%) and NH_4-N (98%) (Figure S2). The major part of conventional pollutants was removed in BC1, whereas the use of BC2 improved further the quality of treated wastewater. As it was expected due to sludge recirculation, the concentrations of activated sludge were almost the same in both bioreactors. On the other hand, the increased organic loading into BC1 resulted in a higher concentration of attached biomass (1023 ± 165 mg L^{-1}) comparing to that observed in BC2 (610±198 mg L^{-1}).

The HMBBR system exhibited significant decreases of all the target compound concentrations in wastewater (Figure 15), resulting in average removals ranging between 40% (4TTR) and 80% (OHBTH).

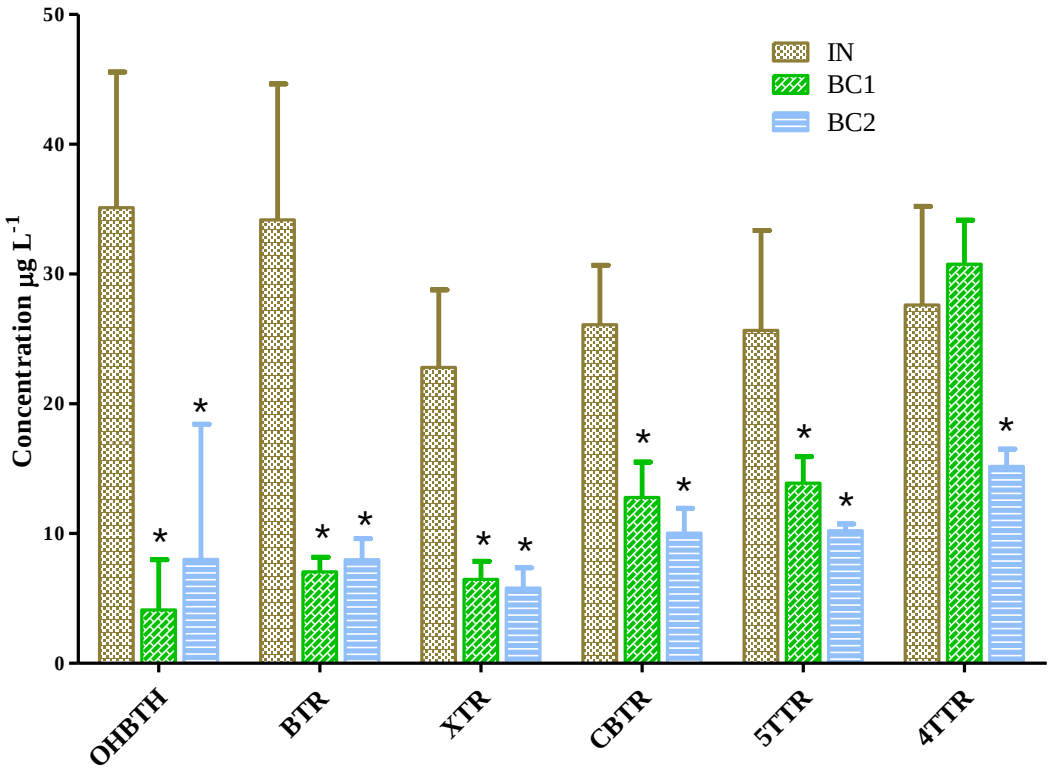


Figure 15: Concentrations (as µg L^{-1}) of target compounds in raw sewage (IN), effluent sewage of the 1st bioreactor (BC1) and effluent sewage of the 2nd bioreactor (BC2) of the HMBBR system (t-bars represent 95% confidence interval; the use of star indicates statistical differences at 95% confidence level from IN sample).

The observed decrease of micropollutants concentration was mainly due to biodegradation (as the compounds are not degraded abiotically in STPs and they are poorly sorbed to biomass). Except for 4TTR, all investigated chemicals were removed in BC1, while the second bioreactor (BC2) did not statistically significantly improve their removal. The removal of most target compounds in BC1 where there was a higher COD concentration indicates the role of co-metabolism in the compounds biodegradation. Concerning 4TTR, it seems that the biomass grown in BC2 had the ability to biodegrade it, whereas this property was not present in BC1. So far, in the literature contradictory results have been reported for biodegradation of 4TTR and 5TTR in AS and MBBR systems, indicating the important role of biomass used and the role of specific microorganisms on their removal (Weiss et al., 2006; Herzog et al., 2014a).

When comparing the removal efficiency of target compounds in the HMBBR system with those previously observed in pure MBBR and AS systems, we can see that the current system achieved

similar or statistically higher elimination for 4 out of 6 examined chemicals (Figure 16). Only OHBTH and 4TTR were removed more efficiently in a pure MBBR system that operated under lower organic loading conditions (0.25 kg m^{-3} d^{-1} in the first stage and 0.05 kg m^{-3} d^{-1} in the second stage) and double HRT. It is worth mentioned that when the performance of the HMBBR system is compared with that of a pure MBBR system operated under similar organic loading and HRT conditions (MBBR-high, Figure 16), a statistically significant increase of removal is observed for 5 out of 6 target compounds, indicating the advantage of the hybrid system on micropollutants removal comparing to a pure MBBR system operated under the same conditions. Finally, the hybrid system achieved statistically higher removal efficiencies for XTR and 5TTR and similar removal for the other compounds comparing to an AS system operated at the double HRT and the same concentration of suspended biomass (Figure 16). In a previous study, Di Trapani et al. (2010) reported that HMBBR systems can achieve similar performance in terms of organic and nitrogen removal as a traditional AS system operating at lower hydraulic loading, however, this it is the first time that this is described for micropollutants removal. The efficient performance of a HMBBR system under higher loadings comparing to traditional AS systems could significantly decrease the operational costs of STPs as it is known that the energy consumption for aeration of AS tanks contribute to 40-75% of the total energy requirements in STPs (Mamais et al., 2015).

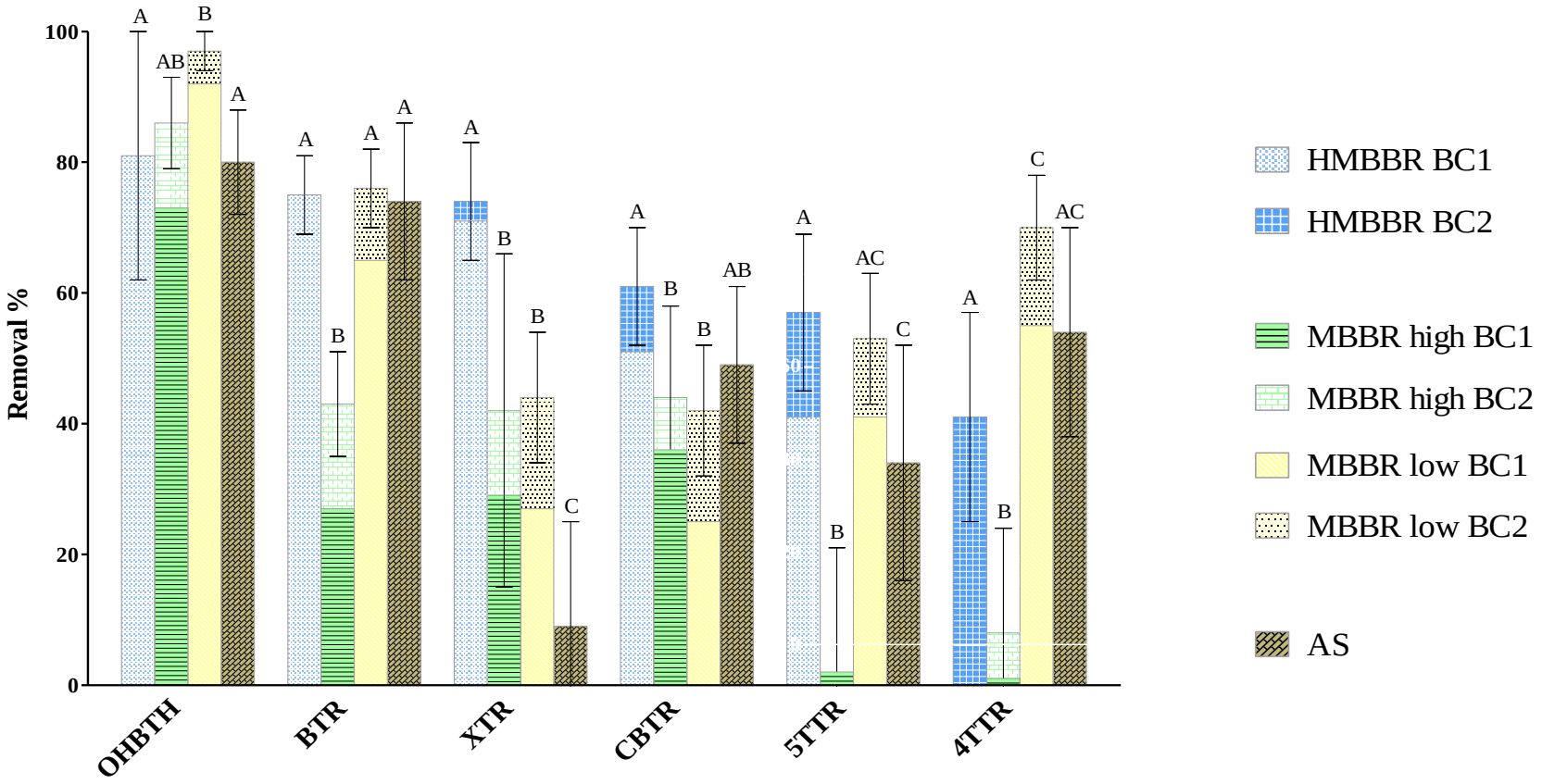


Figure 16: Percent removal of target compounds in the AS, MBBR and HMBBR lab-scale systems (in BC1 and BC2). One way ANOVA analysis followed by Tukey's multiple comparison test, for 95% confidence intervals, was performed in order to determine statistically different means (indicated with letters).

Biodegradation constants were calculated for each type of biomass (Figure 17, Table S4). As can be seen in Figure 17, different normalized biodegradation constants were calculated for the two types of biomass contained in the same bioreactor, indicating the significant role of both types of biomass on the removal of this group of micropollutants in a HMBBR system. Specifically in BC1, OHBTH and BTR were biodegraded more rapidly by activated sludge, whereas the opposite was observed for CBTR. Additionally in BC2, higher k_{bio} was calculated for OHBTH, BTR, XTR and CBTR by attached biomass.

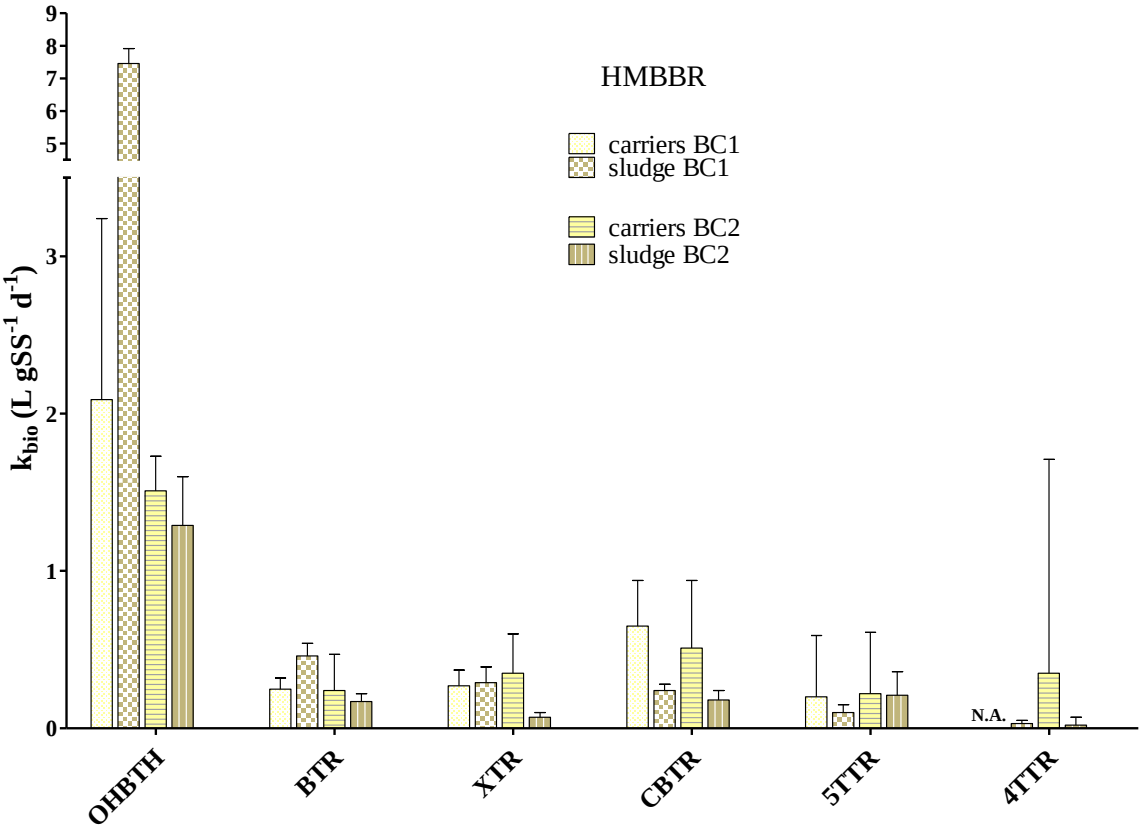


Figure 17: Biodegradation constants (k_{bio}, as L g_{SS}^{-1} d^{-1}) for the HMBBR system calculated in batch experiments with activated sludge and attached biomass from BC1 and BC2.

The removal of target compounds in the HMBBR system was predicted using batch biodegradation kinetics and a modification of Equations 5 and 6 (Figure 18). Despite the underestimation of removal efficiencies that was observed for some of the target compounds especially in the first reactor (BC1), the applied model described sufficiently the order of removal of studied micropollutants in HMBBR

system. Concerning the contribution of different types of biomass to the target compounds removal, it seems that biodegradation by AS occurring in BC1 is the major mechanism for OHBTH, BTR and XTR. Both biocarriers and AS of BC1 and BC2 contribute significantly on biodegradation of CBTR and 5TTR, whereas the attached biomass on biocarriers of BC2 has critical role for 4TTR biodegradation. As it was expected due to the hydrophilicity of these compounds, the role of sorption in their removal is of minor importance.

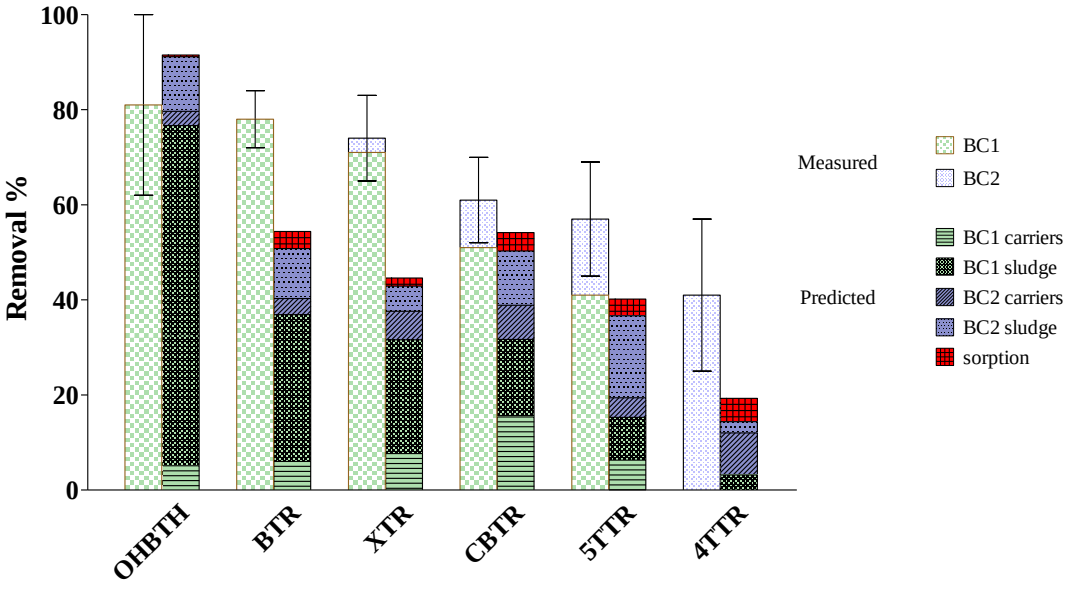


Figure 18: Measured and predicted removal of target compounds in HMBBR system. The contribution of different types of biomass (carriers and sludge) and different mechanisms on their removal is also shown (for predicted removal, the biodegradation with BC1 and BC2 carriers and sludge as well as the sorption on sludge were determined).

Concerning transformation products, twenty two transformation products were tentatively identified in total with mass accuracy ±5 ppm. The m/z range of the candidate TPs ranged from 132.0567 (TP14) to 245.9536 (TP22). For the majority of the candidates, retention times showed the formation of more polar TPs than the parent compounds. A distinctive time trend (absent in the blank, increasing peak over incubation time) was observed for all candidate TPs. All information about TPs is summarized in Table S5. As identification confidence in HR-MS is sometimes difficult to communicate in an accurately way (Bletsou et al. 2015), in the present work we used the levels of identification confidence proposed by Schymanski et al. (2014). BTR presented the higher degree of biotransformation compared to the

other BTRs, as previously reported by Huntscha et al. (2014). Five candidate TPs were found in positive mode (TP1-TP5) and 4 more (TP6-TP9) in negative mode. Hydroxylation was the dominant reaction mechanism followed by oxidation and methylation. Previously reported TPs for BTR (Liu et al., 2011; Huntscha et al., 2014) were among the tentatively identified TPs (TP1-TP7, TP9). In total, five TPs (TP3-TP7) were identified by library spectrum match and the records from the online mass spectra database, MassBank, were reported. Two TPs (TP2 and TP8) were tentatively identified and probable structures were proposed. TP1 (1-OH BTR) was confirmed by a reference standard and for TP9 an unequivocal molecular formula was reported (identification level 1 and 4, respectively; Schymanski et al., 2014). Biotransformation of 4TTR showed 5 candidate TPs (TP10-TP14). Hydroxylation and oxidation were found to be the most probable reaction mechanisms for the formation of the TPs. In positive mode only TP10 (C7H5N3O2) was identified with a tentative structure that is illustrated in Table S5. In negative mode, 4 more TPs were identified. Hydroxylation of the benzene ring was identified for TP14 whereas monohydroxylation of the methyl group were identified for TP13. Both hydroxylation and oxidation reactions were involved in formation of TP11-TP12. For TP12 the probable structure of 4-COOH BTR was proposed by a library spectrum match (Id. level 2a). 5TTR degradation revealed the formation of 3 candidate TPs (TP15-TP17). TP15 was identified to be 5-COOH BTR by a library spectrum match (Id. level 2a). The tentative structure of TP16 (C7H7N3O) corresponds to monohydroxylation, whereas TP17 (C7H7N3O2) corresponds to a dihydroxylation of the benzene ring (ident. level 3). To our knowledge, biodegradation products of XTR has not been studied before, and this is the first report of its biotransformation products. Two candidate TPs (TP18-TP19) were found for XTR and tentative structures were proposed (Id. level 3). TP18 (C8H7N3O2) corresponds to the formation of carboxylic acid XTR, while TP19 (C8H9N3O) indicates either the monohydroxylation of a methyl group or monohydroxylationof the benzene ring of XTR, which was detected in both positive and negative ionization mode. CBTR did not show any potential TP according to the screened database either in positive or negative ionization mode. Finally, OHBTH has also not been studied before, and this is the first report of its biotransformation products. Three candidate TPs (TP20-TP22) were identified and tentative structures were proposed for OHBTH (Id. level 3). TP20 of OHBTH (C8H7NO2S) indicates methoxylation of the benzene ring, whereas the candidate TPs in negative mode TP21 (C7H5NO2S) and TP22 (C7H5NO5S2) correspond to a

hydroxylation of the benzene ring followed by the formation of a sulfonic ester in one of the two hydroxyl groups, respectively.

4. Conclusions and Future Research

4.1. Conclusions

This study investigated the fate of five BTRs and one BTH during biological wastewater treatment. The experimental approach included a) batch experiments, b) continuous flow experiments and c) modeling of obtained results for the prediction of the micropollutants fate. The more important results of this study are briefly presented.

Batch experiments

- Sorption of target compounds onto AS is of minor importance; therefore, they are mainly encountered in the dissolved phase in bioreactors. Sorption constants ranged between 87 (XTR) and 220 L Kg^{-1} (BTR).
- No abiotic transformation occurred to the target compounds in the typical sterilized AS environment.
- Biodegradation half lives varied from 0.25 to 2 days for BTR, XTR, CBTR and OHBTH when treated with AS, at environmental level concentrations (initial concentration: 30 μg L^{-1}).
- The availability of easily degradable organic compounds strongly influenced the removal of the target compounds. Biodegradation seems to occur due to co-metabolism by microorganisms that use either molecular oxygen or nitrates as electron donors and scavenge for a wide range of carbon sources.
- The two SRTs tested for AS (18d and 8d) seemed to have no influence on the removal of target compounds.
- Different biodegradation constants were calculated, depending on the type of biomass (AS or biofilm) and the conditions under which the biomass was developed. Therefore, it seems that there are specific groups and types of microorganisms responsible for the degradation of target compounds.

Continuous flow experiments

- All compounds were removed to some extent in all lab scale systems tested.
- The general removal trend in all the systems was higher for OHBTH and then the other compounds followed: BTR > XTR > CBTR > 5TTR > 4TTR.
- The HMBBR and the MBBR low loaded system presented the higher removal rates. Less efficient were AS and MBBR systems, operating both under the same HRT and organic loading conditions.
- High fluctuations were observed in the removal of 4TTR and 5TTR the among systems.
- The biomass developed in the MBBR system had greater capacity for biodegradation, especially when operated under low organic loading.
- The biomass presented different properties regarding removal, depending on the long-term operational parameters of the systems that led to the development of a different microbial community in each system.
- During the biodegradation experiments with biomass from the HMBBR system, twenty-two transformation products were tentatively identified with hydroxylation, oxidation and methylation being the main reaction mechanisms.
- The HMBBR was the most efficient among the tested systems, regarding the removal rate of target compounds and the required HRT.

Modelling and Prediction

- Partial removal of the investigated compounds is expected in full-scale STPs during biological wastewater treatment and this mainly occurs due to biodegradation in aerobic bioreactors, while elimination due to sorption is expected to be minor.
- The higher concentration of biomass seems to be able to increase removal in a large STP.

- The biodegradation constants calculated in batch experiments with biomass from an STP are adequate for the prediction of removal in the same STP.
- In small-scale systems, the biodegradation constants could not precisely predict removal when batch experiments were conducted at different time intervals. Therefore, the biomass characteristics can rapidly change, influencing the overall removal.

4.2. Future Research

Based on the results of this study and on the questions that emerged during this work, some points for future research are proposed.

Since all the target compounds are only partially removed during biological treatment, further investigation for their tertiary removal is needed. This kind of research is of great importance for STPs that dispose treated wastewater into surface water that could be used as a drinking water source. Treatment with activated carbon or application of ozonation could be investigated for further removal of BTRs and BTHs from treated sewage.

As both AS and biofilm seemed to have a satisfactory capacity in removing part of the micropollutants from wastewater, more work should be done by testing different types of systems. As the AS and HMBBR seemed to remove a large part of the micropollutants from incoming wastewater due to AS and co-metabolic action, these systems could be used as the first stage of a treatment system (operated at a relatively low HRT). This reactor or sequence of reactors could be followed by a pure MBBR, operated at higher HRTs, whereas the lack of easily degradable compounds would lead to the formation of a competitive biofilm that could have advantages in removing persistent micropollutants. This type of system could be tested for the removal of different groups of micropollutants, at a long term operation schedule. This set-up could also be expected to adsorb peak COD loads and assure a high quality effluent.

Furthermore, the introduction of anoxic MBBR reactors could be examined in order to test the potency of biofilm developed under anoxic conditions in removing the target compounds. Though aerobic conditions were preferable for the removal of the examined compounds, there is no information on the potency of anoxic MBBRs upon removal.

As proved in all the experiments, the presence of easily degradable organic substances enhanced biodegradation of the target compounds. Experiments could be conducted with different organic

substrates in order to determine the role of the organic substrate composition in biodegradation of micropollutants.

Concerning fluctuation in removal efficiencies of the treatment systems tested, research should be carried out on how microbial diversity influences the biodegradation of target compounds. Biodegradation experiments, followed by microorganism identification (possibly with FISH) could provide important information on the types of microorganisms that are responsible for each compound decomposition. This could lead to the design and operation of bioreactors with specific microbial diversity (by inoculating them with specific strains) that could achieve maximum biological removal.

Regarding TTR, the ability to identify the two isomers (4TTR and 5TTR) gave important information for their different trends in removal. Further research is needed for the determination of the microorganisms responsible for each isomer removal, as their elimination seems to be directly associated.

Further investigation could focus on the different transformation by-products that are produced, depending on the type of biomass involved in the biodegradation.

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6. Supplementary Materials

Table S1: Model's sensitivity concerning the total removal of target compounds during activated sludge process in typical STPs operating at SRT of 8 d and 18 d (A: prediction based on the experimentally determined biodegradation constants and MLSS concentration of 3000 mg L^{-1}. B: prediction based on the experimentally determined biodegradation constants and MLSS concentration of 5000 mg L^{-1}. C: prediction based on biodegradation constants higher by 20% comparing to those experimentally determined and MLSS concentration of 3000 mg L^{-1}. D: prediction based on biodegradation constants lower by 20% comparing to those experimentally determined and MLSS concentration of 3000 mg L^{-1}).

Compound	Total Removal (%) in a STP operating at a SRT of 18 d				Total Removal (%) in a STP operating at a SRT of 8 d			
	A	B	C	D	A	B	C	D
BTR	29	40	32	25	24	34	27	21
CBTR	39	52	44	34	29	41	33	25
XTR	40	53	44	35	30	41	34	25
OHBTH	45	58	49	40	37	50	41	32

Table S2: Operational parameters of continuous flow systems, during acclimatization and loading with target compounds: Activated Sludge (AS, HRT 26.4 ± 2.4 hours), Biocarriers under low loading conditions (MBBR-low, HRT 26.4 ± 3.6 hours for each reactor) and Biocarriers under high loading conditions (MBBR-high, HRT 10.8 ± 1.2 hours for each reactor).

Activated Sludge System													
Continuous flow system	Days of operation		MLSS (mg L^{-1})	TSS (mg L^{-1})	pH		Removal %					
							COD dissolved			NH_4-N		
			AB[1]	Out[2]	AB[1]	Out[2]	AB			AB		
AS (n = 16)	31		2370 (±590)	11 (±13)	7.2 (±0.4)	7.3 (±0.6)	90 (±7)			93 (±12)		
Moving Bed Bioreactor System												
Continuous flow system	Days of operation	Attached Biomass (mg L^{-1})		MLSS (mg L^{-1})	MLSS (mg L^{-1})	pH		Removal %					
								COD dissolved			NH_4-N		
		BC1[3]	BC2[4]	BC1[3]	BC2[4]	BC1[3]	BC2[4]	BC1[3]	BC2[4]	Total[5]	BC1[3]	BC2[4]	Total[5]
MBBR-low (n = 15)	45	726	100	195 (±81)	131 (±89)	7.0 (±0.5)	6.8 (±0.9)	81 (±13)	42 (±26)	86 (±11)	78 (±29)	84 (±23)	93 (±13)
MBBR-high (n = 11)	45	1079 (±715)	312 (±108)	138 (±68)	124 (±68)	7.4 (±0.2)	7.2 (±0.3)	72 (±11)	67 (±21)	91 (±7)	73 (±24)	87 (±21)	95 (±7)

[1]AB: aerobic bioreactor with activated sludge; [2]Out: treated wastewater; [3]BC1: bioreactor with biocarriers1; [4]BC2: bioreactor with biocarriers2; [5]Total: Total Removal in BC1 and BC2

Table S3. Biodegradation constants calculated during batch experiments with suspended and attached biomass (AS and MBBR), under low and high COD concentrations (average values and standard deviation), from **Paper B**.

Biodegradation rate constant, k (d^{-1})													
Experiment	COD	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.
		BTR		**4TTR**		**5TTR**		**CBTR**		**XTR**		**OHBTH**	
BC1	low	**0.66**	0.21	**N.A.**	N.A.	**N.A.**	N.A.	**0.41**	0.37	**0.22**	0.14	**4.74**	1.62
BC1	high	**0.98**	0.33	**0.15**	0.12	**N.A.**	N.A.	**0.48**	0.56	**0.49**	0.61	**3.43**	0.44
BC2	low	**0.90**	0.26	**0.20**	0.08	**0.27**	0.16	**0.64**	0.30	**0.43**	0.12	**1.82**	1.06
BC2	high	**2.03**	2.22	**N.A.**	N.A.	**N.A.**	N.A.	**2.43**	1.64	**0.53**	1.46	**1.78**	1.17
AB	low	**0.50**	0.11	**N.A.**	N.A.	**0.11**	0.09	**0.90**	0.13	**0.58**	0.12	**2.41**	0.78
AB	high	**1.11**	0.32	**0.02**	0.01	**0.21**	0.16	**1.07**	0.74	**0.68**	0.08	**3.36**	0.94
Pseudo first-order biodegradation rate constant, k_{bio}(L g_{SS}^{-1} d^{-1})													
Experiment	COD	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.
		BTR		**4TTR**		**5TTR**		**CBTR**		**XTR**		**OHBTH**	
BC1	low	**0.58**	0.18	**N.A.**	N.A.	**N.A.**	N.A.	**0.36**	0.33	**0.17**	0.10	**3.84**	1.31
BC1	high	**0.86**	0.29	**0.12**	0.10	**N.A.**	0.14	**0.43**	0.50	**0.37**	0.46	**2.78**	0.35
BC2	low	**2.46**	0.71	**0.54**	0.20	**0.72**	0.42	**1.77**	0.82	**1.11**	0.31	**4.86**	2.84
BC2	high	**5.58**	6.08	**N.A.**	N.A.	**N.A.**	N.A.	**6.72**	4.54	**1.39**	3.81	**4.77**	3.14
AB	low	**0.31**	0.06	**N.A.**	N.A.	**0.06**	0.05	**0.57**	0.08	**0.28**	0.06	**1.30**	0.42
AB	high	**0.68**	0.19	**0.01**	0.01	**0.12**	0.09	**0.67**	0.47	**0.32**	0.04	**1.82**	0.51

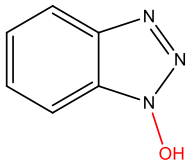
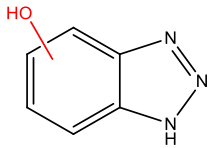
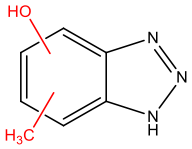
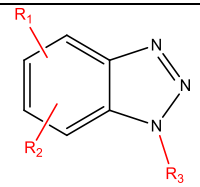
AB: Aerobic Bioreactor with activated sludge collected from Mytilini's STP, BC1: Bioreactor with Biocarriers 1 collected from MBBR high, BC2: Bioreactor with Biocarriers2 collected from MBBR high, COD low: initial concentration 28 (±15) mg L^{-1}, COD high: initial concentration 272 (±107) mg L^{-1}

Table S4: Biodegradation constants calculated during batch experiments with biocarriers and activated sludge (AS) from 1st bioreactor (BC1) and 2nd bioreactor (BC2) in HMBBR system(average values and standard deviation), from **Paper C**.

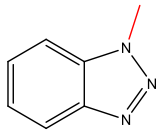
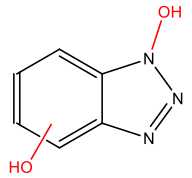
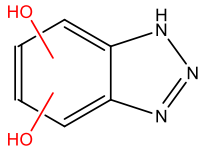
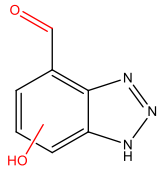
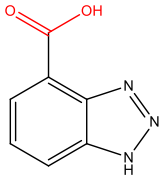
Biodegradation rate constant, k (d^{-1})																			
Experiment	type	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2
		OHBTH			**BTR**			**XTR**			**CBTR**			**5TTR**			**4TTR**		
BC1[1]	carriers	**2.43**	1.34	0.902	**0.29**	0.08	0.971	**0.31**	0.11	0.950	**0.75**	0.34	0.935	**0.23**	0.45	0.392	N.A.		
BC1[2]	sludge	**25.22**	1.57	0.985	**1.54**	0.26	0.984	**0.98**	0.33	0.925	**0.81**	0.13	0.991	**0.34**	0.17	0.914	**0.09**	0.06	0.669
BC2[3]	carriers	**1.17**	0.17	0.985	**0.19**	0.18	0.742	**0.27**	0.20	0.637	**0.40**	0.33	0.774	**0.17**	0.30	0.421	**0.27**	1.05	0.735
BC2[4]	sludge	**4.84**	1.17	0.997	**0.63**	0.20	0.916	**0.26**	0.12	0.921	**0.68**	0.23	0.959	**0.79**	0.57	0.841	**0.08**	0.17	0.897
Pseudo first-order biodegradation rate constant, k_{bio} (L g_{SS}^{-1} d^{-1})																			
Experiment	type	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2
		OHBTH			**BTR**			**XTR**			**CBTR**			**5TTR**			**4TTR**		
BC1[1]	carriers	**2.09**	1.15	0.902	**0.25**	0.07	0.971	**0.27**	0.10	0.950	**0.65**	0.29	0.935	**0.20**	0.39	0.392	N.A.		
BC1[2]	sludge	**7.46**	0.46	0.985	**0.46**	0.08	0.984	**0.29**	0.10	0.925	**0.24**	0.04	0.991	**0.10**	0.05	0.914	**0.03**	0.02	0.669
BC2[3]	carriers	**1.51**	0.22	0.985	**0.24**	0.23	0.742	**0.35**	0.25	0.637	**0.51**	0.43	0.774	**0.22**	0.39	0.421	**0.35**	1.36	0.735
BC2[4]	sludge	**1.29**	0.31	0.997	**0.17**	0.05	0.916	**0.07**	0.03	0.921	**0.18**	0.06	0.959	**0.21**	0.15	0.841	**0.02**	0.05	0.897

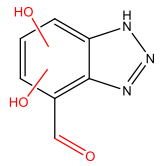
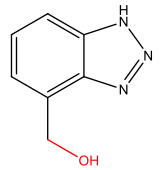
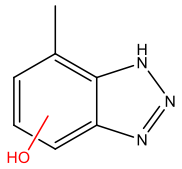
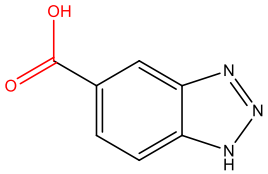
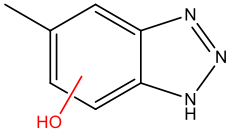
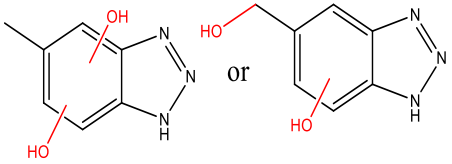
[1]Experiments with biocarriers from BC1 were conducted with COD initial concentration of 203 mg L^{-1}; [2]Experiments with AS from BC1 were conducted with COD initial concentration of 223 mg L-1; [3]Experiments with biocarriers from BC2 were conducted with COD initial concentration of 28 mg L^{-1}; [4]Experiments with AS from BC2 were conducted with COD initial concentration of 59 mg L^{-1}.

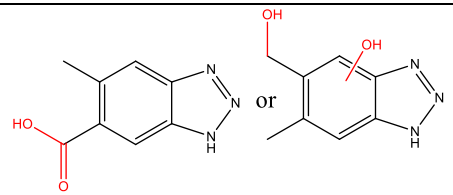
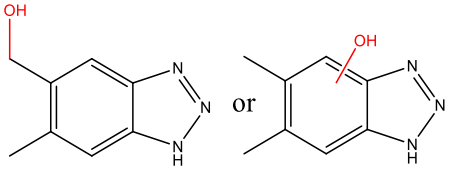
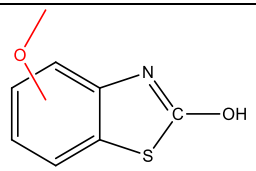
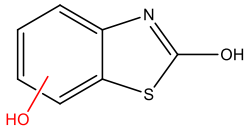
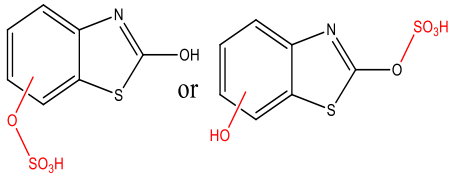
Table S5: Description of candidate TPs observed in batch biodegradation experiments with biomass from HMBBR system

Parent compound	TP	ESI polarity/ Precursor ion	m/z	Rt(min)	Molecular Formula	Tentative Structures	Id. Level (MassBank Record)	Time trend[a]	Reported in Literature
BTR	TP1	$[M+H]^+$	136.0505	3.8	$C_6H_5N_3O$		1	↗	Huntscha et al., 2014
	TP2	$[M+H]^+$	136.0505	4.1	$C_6H_5N_3O$		3	↗	Huntscha et al., 2014
		$[M-H]^-$	134.0360	4.0				↗	
	TP3	$[M+H]^+$	150.0662	5.1	$C_7H_7N_3O$		3 (ETS00101)	↗	Huntschaetal., 2014 Liu et al., 2011
	TP4	$[M+H]^+$	178.0611	3.5	$C_8H_7N_3O_2$		3 (ETS00108)	↗	Huntscha et al., 2014
	TP5	$[M+H]^+$	178.0611	4.2			3 (ETS00109)	↗	Huntscha et al., 2014

R_1, R_2, R_3: H, CH_3, COOH

	TP6	$[M-H]^-$	132.0567	3.7	$C_7H_7N_3$		2a (ETS00115)	↗	Huntschaetal., 2014 Liu et al., 2011
	TP7	$[M-H]^-$	150.0309	1.6	$C_6H_5N_3O_2$		3 (ETS00103)	↗	Huntschaetal., 2014
	TP8	$[M-H]^-$	150.0309	3.1	$C_6H_5N_3O_2$		3	↗	
	TP9	$[M-H]^-$	182.0207	1.2	$C_6H_5N_3O_4$	-	4	↗	Huntschaet al., 2014
4TTR	TP10	$[M+H]^+$	164.0455	4.1	$C_7H_5N_3O_2$		3	↗	Huntscha et al., 2014
		$[M-H]^-$	162.0309	3.2					
	TP11	$[M-H]^-$	162.0309	2.3	$C_7H_5N_3O_2$		2a (ETS00107)	↗	Huntscha et al., 2014

	TP12	[M-H]$^-$	178.0258	1.3	$C_7H_5N_3O_3$		3	↗	
	TP13	[M-H]$^-$	148.0516	3.9	$C_7H_7N_3O$		3 (ETS00102)	↗↘	Huntscha et al., 2014
	TP14	[M-H]$^-$	148.0516	4.7	$C_7H_7N_3O$		3 (ETS00102)	↗↘	Huntscha et al., 2014
5TTR	TP15	[M+H]$^+$	164.0455	3.7	$C_7H_5N_3O_2$		2a (ETS00121)	↗	Huntscha et al., 2014
		[M-H]$^-$	162.0309	3.7					
	TP16	[M+H]$^+$	150.0662	4.6	$C_7H_7N_3O$		3 (ETS00102)	↗	Huntscha et al., 2014
	TP17	[M-H]$^-$	164.0466	2.9	$C_7H_7N_3O_2$		3	↗	

XTR	TP18	$[M+H]^+$	178.0611	3.8	$C_8H_7N_3O_2$		3	↗	
	TP19	$[M+H]^+$	164.0818	5.4	$C_8H_9N_3O$		3	↗	
		$[M-H]^-$	162.0673	4.9					
CBTR			-						
OHBTH	TP20	$[M+H]^+$	182.0270	3.1	$C_8H_7NO_2S$		3	↗	
	TP21	$[M-H]^-$	165.9968	5.8	$C_7H_5NO_2S$		3	↗	
	TP22	$[M-H]^-$	245.9536	4.2	$C_7H_5NO_5S_2$		3	↗↘	

[a]The symbols(↗) and (↘)in time trend column indicate whether there is an increase or decrease in formation of a specific TP. In red it is indicated the transformation of the parent compound.

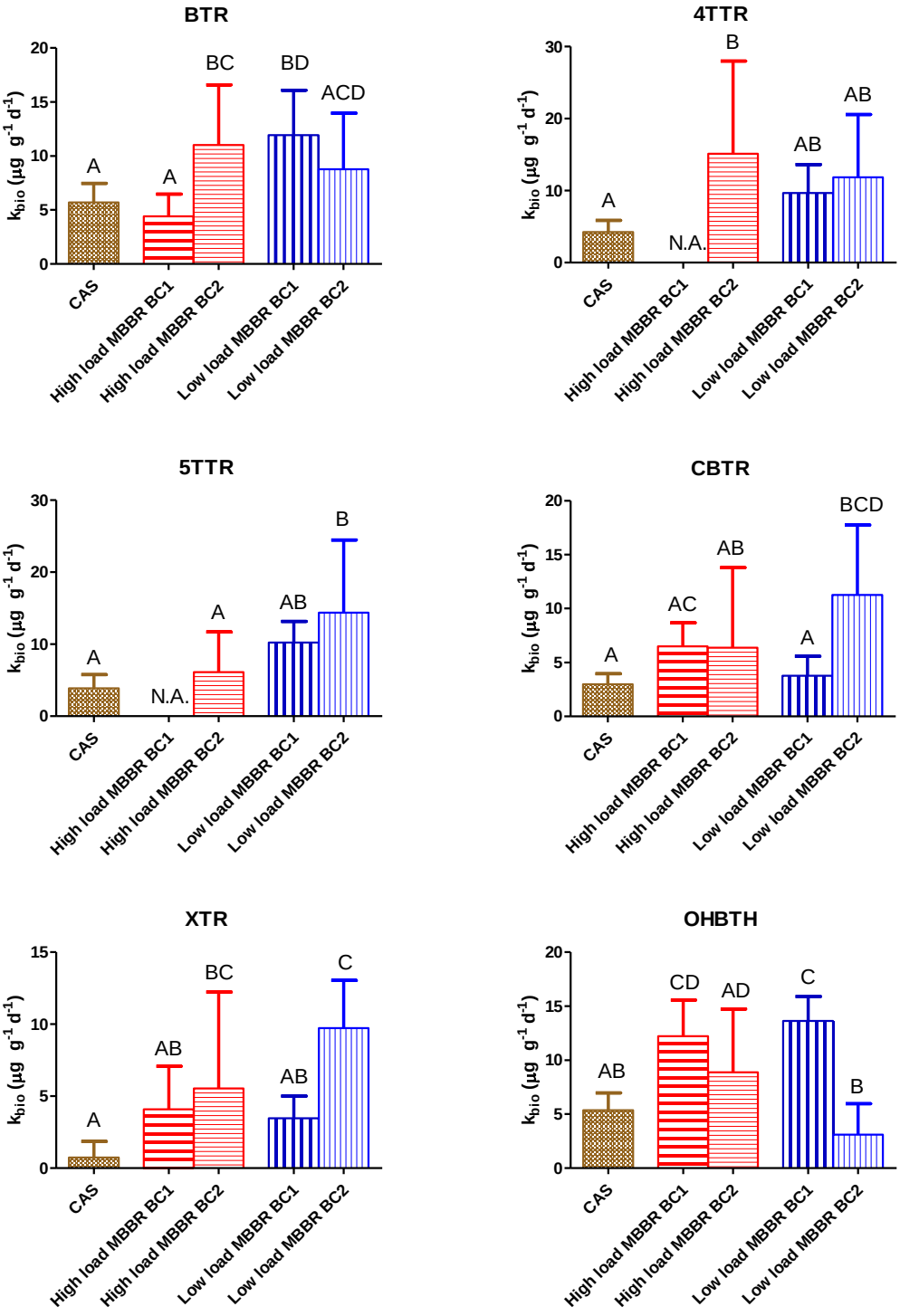


Figure S1: Mass of micropollutants removed per mass of biomass and day during continuous flow experiments with Activated Sludge (AS), Biocarriers under low loading conditions (MBBR-low) and Biocarriers under high loading conditions (MBBR-high). Results are given for each bioreactor (BC1 and BC2), separately (different letters indicate statistical differences at 95% confidence level; t-bars represent 95% confidence interval).

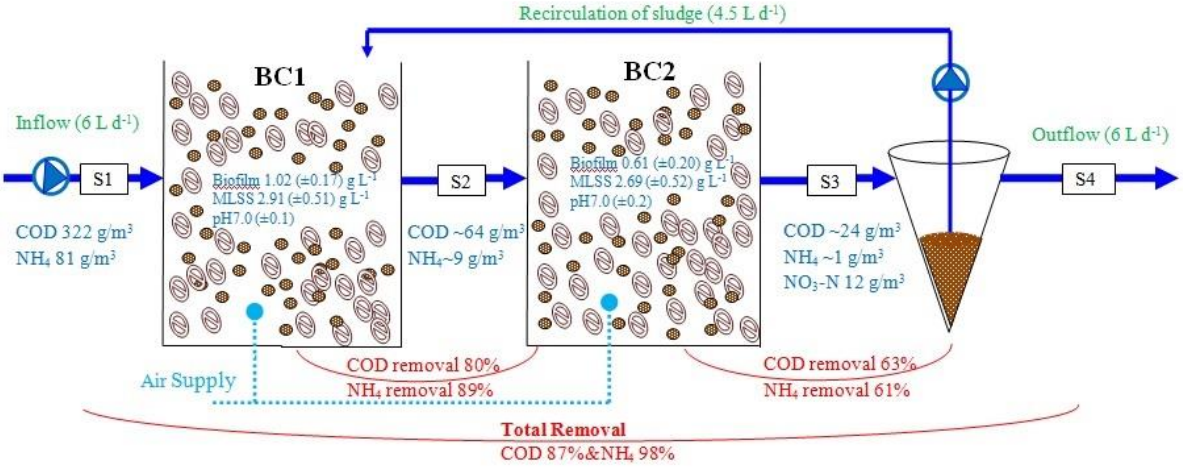


Figure S2: Schematic representation, operational characteristics and performance of the HMBBR system used in this study (HRT was equal to 12.4 ± 0.6 h for each reactor; sampling points are indicated with an S).

Paper A

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Sorption and biodegradation of selected benzotriazoles and hydroxybenzothiazole in activated sludge and estimation of their fate during wastewater treatment

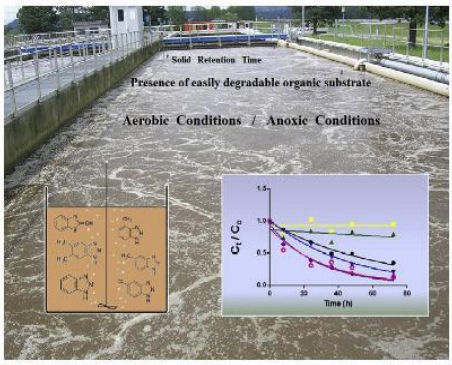
Aikaterini A. Mazioti [a], Athanasios S. Stasinakis [a,*], Georgia Gatidou [a], Nikolaos S. Thomaidis [b], Henrik R. Andersen [c]

[a] Department of Environment, University of the Aegean, Mytilene, Greece
[b] Department of Chemistry, National and Kapodistrian University of Athens, Panepistimioupolis Zografou, 15771 Athens, Greece
[c] Department of Environmental Engineering, Technical University of Denmark, Miljøvej, B 113, 2800 Kgs., Lyngby, Denmark

HIGHLIGHTS

- The sorption and biodegradation of selected BTRs and OHBTH was studied.
- Anoxic, aerobic conditions, SRT and organic substrate were investigated.
- BTR, CBTR, XTR and OHBTH were biodegraded under aerobic, anoxic conditions.
- With one exception, Sludge Retention Time did not affect biodegradation kinetics.
- Partial removal of investigated compounds expected in STPs, mainly by biodegradation.

GRAPHICAL ABSTRACT



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ABSTRACT

Biodegradation of benzotriazole (BTR), 5-chlorobenzotriazole (CBTR), xylytriazole (XTR), 4-methyl-1H-benzotriazole (4TTR), 5-methy-1H-lbenzotriazole (5TTR) and 2-hydroxybenzothiazole (OHBTH) was studied in activated sludge batch experiments under aerobic and anoxic conditions, presence of organic substrate and different sludge residence times (SRTs). Their sludge-water distribution coefficients were also calculated in sorption experiments and ranged between 87 and 220 L kg^{-1}. Significant biodegradation of BTR, CBTR, XTR and OHBTH was observed in all biotic experiments. Half-life values ranged between 23 and 45 h (BTR), 18 and 47 h (CBTR), 14 and 26 h (XTR), 6.5 and 24 h (OHBTH). The addition of substrate did not suppress biodegradation kinetics; whereas in some cases accelerated biodegradation of microcontaminants. Except for CBTR, no effect of SRT on biodegradation constants was observed. Prediction of micropollutants removal in Sewage Treatment Plants (STPs) indicated that they will be partially removed, mainly due to aerobic biodegradation. Higher removal is expected at STPs operating at higher SRT and higher suspended solids concentrations.

* Corresponding author. Tel.: +30 22510 36257; fax: +30 22510 36206.
E-mail address: astas@env.aegean.gr (A.S. Stasinakis).

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1. Introduction

Benzotriazoles (BTRs) and benzothiazoles (BTHs) are two classes of emerging contaminants that have been extensively detected in the aquatic environment, worldwide (Loos et al., 2009; Nödler et al., 2014). BTRs consist of a benzene ring fused with a triazole ring, they are soluble in water, slightly basic (pK_a 8.2–8.8) and have a weak tendency to sorb onto organic matter (Weiss et al., 2006). They are widely used in several industrial applications for protection of metal mechanical parts, as well as in everyday products and dishwashing detergents (Janna et al., 2011; Kiss and Fries, 2012). In the case of BTHs, a benzene ring is fused with a thiazole ring. These compounds are also polar, they are used in tire and rubber manufacturing industries and they are found in biocides, drugs and food flavors (Llompart et al., 2013).

During the last decade, the occurrence of BTRs and BTHs in Sewage Treatment Plants (STPs) has been documented around the world (Reemtsma et al., 2010; Liu et al., 2012; Stasinakis et al., 2013). The concentrations of these compounds in raw sewage vary from some hundred ng L^{-1} to some tens μg L^{-1}, while they are partially removed during conventional wastewater treatment (Weiss et al., 2006; Stasinakis et al., 2013). Despite the frequent detection of BTRs and BTHs in STPs, so far, there is little information on their fate in activated sludge processes and the role of sorption and biodegradation on their removal. Previous research has mainly focused on benzotriazole (BTR), 4-methyl-1H-benzotriazole (4TTR), and 5-methy-1H-lbenzotriazole (5TTR), while in most cases the experiments have been conducted at much higher concentrations than that is found in wastewater. Specifically, in experiments with activated sludge and initial concentration of target compounds equal to 1 mg L^{-1}, Liu et al. (2011) studied the biodegradation potential of BTR, 5TTR and 5-chlorobenzotriazole (CBTR) under aerobic conditions and proposed their biotransformation pathways. In a recent study, Huntscha et al. (2014) investigated the biotransformation of BTR, 4TTR, and 5TTR under aerobic conditions (initial concentrations: 0.5–2.4 mg L^{-1}), determined their half-lives and identified the major biotransformation products. Finally, Herzog et al. (2014a, b) studied the removal efficiency of BTR, 4TTR and 5TTR under different experimental conditions at initial concentrations ranging between 0.2 and 34 mg L^{-1}, and reported that sludge acclimatization enhanced biodegradation of some compounds. To the best of our knowledge, no data is available for the fate of xylytriazole (XTR) and 2-hydroxybenzothiazole (OHBTH) in activated sludge processes. On the contrary, it is known that the biodegradation of micropollutants during activated sludge process is affected by factors such as the redox conditions, the sludge residence time (SRT) and the presence of supplementary substrate (Joss et al., 2004; Stasinakis et al., 2009; Falås et al., 2012; Vasiliadou et al., 2014). Except for BTR, 4TTR and 5TTR (Herzog et al., 2014a,b), no data is available for the effects of these parameters on biodegradation of BTRs and BTHs. Moreover, there is a lack of data for sorption of these compounds to sludge, as well as for the contribution of biodegradation and sorption on their removal from STPs.

Therefore, the main objectives of this study were to investigate biodegradation and sorption potential of five BTRs (BTR, CBTR, XTR, 5TTR and 4TTR) and 2-hydroxybenzothiazole (OHBTH) in activated sludge processes (Table S1). Batch biodegradation experiments were conducted at target compounds concentration levels similar to those reported in the literature for domestic wastewater (ppb level). The effect of aerobic and anoxic conditions, presence of easily degradable substrate and SRT on BTRs and OHBTH biotransformation kinetics was investigated. Additionally, batch experiments were conducted to calculate sludge-water distribution coefficients (K_d) of target compounds. Finally, a model was developed to predict the removal of target compounds during activated sludge processes and to investigate the contribution of biodegradation and sorption to their elimination.

2. Materials and methods

2.1. Analytical standards and reagents

Analytical standards of XTR and CBTR were supplied by Sigma–Aldrich (USA). BTR was purchased by Merck (Germany), 4TTR by Fluka (Switzerland), 5TTR by Acros Organics (Belgium) and OHBTH by Alfa Aesar (USA). Stock solutions of individual compounds were prepared in methanol (MeOH) at 1000 mg L^{-1} and kept at −18 °C. MeOH (HPLC-MS grade) and acetonitrile (ACN, HPLC grade) were purchased by Merck (Germany) and Fisher (USA), respectively. The solid phase extraction (SPE) cartridges used for samples' clean-up were Strata-X (33u Polymeric Reversed Phase, 200 mg/6 ml) and they were supplied by Phenomenex (USA). HPLC grade water was prepared in the laboratory using a MilliQ/MilliRO Millipore system (USA). Ultra-pure HCl (32%) was purchased by Merck (Germany).

2.2. Sorption experiments

Sorption experiments were conducted with frieze-dried sludge and tap water and were based on previous studies (Andersen et al., 2005; Arvaniti et al., 2014). In brief, activated sludge was washed three times using tap water, centrifuged to remove water soluble constituents and frozen at −18 °C for 24 h. Afterwards, sludge was gently freeze dried to preserve its structure, sterilized by heating at 103 °C for minimum 3 h and stored at 4 °C.

To determine K_d values of the investigated compounds, batch experiments were conducted for a range of initial concentrations of each compound (10, 40, 80, 150, 300 and 500 μg L^{-1}) to 3 g L^{-1} sludge and 100 mL tap water. Flasks were covered in order to inhibit photodegradation, agitated at 120 rpm on a shaking plate and samples were taken at the end of the experiment (24 h) for analysis of compounds in the water phase. All the experiments were performed at 22.0 ± 1.0 °C, while pH was 7.3 ± 0.2.

2.3. Biodegradation experiments

Activated sludge from a nitrifying municipal STP (STP A, Mytilene, Greece), operating at a SRT of 18 d, was used for most biodegradation experiments. After being collected, biomass was left to settle and the supernatant was rejected and replaced with tap water. Afterwards, sludge was aerated for 48 h and diluted to achieve the desired concentration.

The experimental conditions used in different biodegradation batch experiments (A to G) are presented in Table 1. Experiments were conducted in stoppered glass bottles that were constantly agitated on a shaking plate. The working volume in each reactor was 1 L and the mixed liquor suspended solids (MLSS) concentration 3000 ± 200 mg L^{-1}. The investigated compounds were spiked using methanol solutions to obtain an initial concentration of around 30 μg L^{-1} for each microcontaminant in the reactors. The addition of methanol (100 μL in each reactor) resulted to a theoretical oxygen demand of 120 mg L^{-1}. To quantify biodegradation of micropollutants, homogenized samples of mixed liquor (10 mL) were collected after 0, 8, 24, 36, 48 and 72 h. The concentrations of target compounds were determined in the dissolved and particulate phase using the analytical methods described below.

In aerobic experiments (Experiments A, C, E), dissolved oxygen concentrations higher than 4 mg L^{-1} were achieved by constantly

Table 1
Experimental protocol used in biodegradation batch experiments (initial concentration of target compounds: 30 μg L^{-1}; concentration of mixed liquor suspended solids, MLSS: 3000 ± 200 mg L^{-1}; experiments A to E: 3 replicates, experiments F to G: 1 replicate).

Batch experiments	Constituents	Conditions	Sludge origin
A	Sludge + Target Compounds	Aerobic	STP A[a]
B	Sludge + Target Compounds	Anoxic	STP A
C	Sludge + Target Compounds + Organic Substrate	Aerobic	STP A
D	Sludge + Target Compounds + Organic Substrate	Anoxic	STP A
E	Sludge + Target Compounds	Aerobic	STP B[b]
F	Sterilized Sludge + Target Compounds + Organic Substrate + NaN_3	Aerobic	STP A
G	Sterilized Sludge + Target Compounds + Organic Substrate + NaN_3	Anoxic	STP A

[a] STP A operated at SRT of 18 d.
[b] STP B operated at SRT of 8 d.

supplying air through porous ceramic diffusers. In anoxic experiments (Experiments B, D), the reactors were initially purged with N_2 gas and a solution of $NaNO_3$ was added to provide an initial concentration of NO_3–N equal to 40 mg L^{-1}. To investigate the role of easily degradable substrate on target compounds biodegradation, synthetic wastewater containing peptone, urea, yeast extract, and other micronutrients (Lozada et al., 2004) was added every 24 h in order to achieve chemical oxygen demand (COD) concentration equal to 200 mg L^{-1} in the appropriate flasks. To investigate the role of SRT on target compounds removal, aerobic experiments were also performed using biomass originating from a nitrifying STP that operated at SRT of 8 d (STP B, Athens, Greece). Finally, to investigate the effect of abiotic conditions on target compounds removal, batch experiments were performed with sodium azide (NaN_3, 0.2% w/v) to inactivate the bioactivity (Experiments F and G). Experiments A to E were conducted in triplicate; whereas experiments F to G were conducted without replication (Table 1). In all experiments the temperature was 22.0 ± 0.5 °C, while pH ranged between 7.2 and 8.2.

2.4. Analytical methods

To control the operation of batch reactors, analyses of COD, NH_4–N, NO_3–N and MLSS were periodically performed, according to Standard Methods (APHA, 1998). Moreover, temperature, dissolved oxygen and pH were measured daily in all systems using portable instruments.

For the investigation of target compounds fate, samples were filtered through pre-ashed glass fiber filters (GF-3 Macherey Nagel). Filtrates were collected, acidified to pH 3.0 ± 0.1 and stored at 4 °C until analysis. Filters were oven dried at 60 °C until constant weight and stored at −18 °C. Analysis of target compounds in the dissolved and particulate phase was based on previously developed methods by Asimakopoulos et al. (2013) and included SPE for liquid samples and sonication, followed by SPE clean-up step, for solid samples (Fig. S1).

Chromatographic analysis was performed by a Shimatzu (Japan) LC20-AD prominence liquid chromatographer associated with a SPD-M20A diode array detector (DAD) and a SIL-20AC auto sampler. The column was a Zorbax SB-C18 4.6 mm × 150 mm (5 μm) connected with a Zorbax SB-C18 pre-column (Agilent, USA). The column and pre-column were heated at 35 °C with a CTO-20AC column oven (Shimatzu-Japan). The mobile phase consisted of MilliQ grade water 0.05% acetic acid (solvent A) and ACN (solvent B). Gradient elution was performed as follows: from 25% ACN to 75% ACN in 15 min, hold for 9 min and then decrease to 25% ACN in one minute. The system was equilibrated for 10 min with 25% ACN before each run. The total duration of the separation program was 35 min and the flow rate was 0.5 mL min^{-1}. The DAD was set at measurement wavelengths ranging from 190 to 300 nm, while all compounds were quantified using the signal at 254 nm. The identification of the six compounds in the sample was accomplished on the basis of their retention times and comparing their UV spectrum in the standard solutions and in the samples. A typical chromatogram of target compounds is presented in Fig. S2.

Validation of the analytical methods included analytical methods calibration, determination of limits of detection (LODs), assessment of precision and evaluation of trueness for both dissolved and particulate phase samples (Table S2). Analytical methods calibration was carried out for concentrations ranging from 10.0 to 500 μg L^{-1} and the response of the diode array detector was linear for all target compounds ($R^2 > 0.99$). Satisfactory recoveries and precision of the analytical procedures were achieved. For dissolved samples, the obtained LODs ranged from 17 (BTR) to 125 (CBTR) ng L^{-1}; whereas for particulate samples the LODs varied between 40 (BTR) and 555 ng g^{-1} dry sludge (5TTR) (Table S2).

2.5. Calculations and modeling equations

Sludge-water distribution coefficients, K_d values, (L g^{-1}) of target compounds were estimated from sorption experiments using Eq. (1):

$$K_d = \frac{q_e}{C_e} \quad (1)$$

where q_e is the concentration of target compound in the particulate phase (μg g^{-1}) and C_e is the concentration of target compound in the dissolved phase (μg L^{-1}).

The half-lives of target compounds in aerobic and anoxic biodegradation experiments and the biodegradation rate constants (k) were estimated using first-order kinetics. Pseudo first-order biodegradation rate coefficient, k_{bio}, normalized to mixed liquor suspended solids (L g_{MLSS}^{-1} d^{-1}) was also calculated for each biodegradation experiment using Eq. (2) (Ziels et al., 2014).

$$\ln\frac{C_t}{C_0} = -k_{bio}\left(\frac{MLSS}{1 + K_d MLSS}\right) \times t \quad (2)$$

where C_t and C_0 are the total (dissolved + particulate) target compound concentrations in batch experiment at time t and $t = 0$, respectively, (μg L^{-1}).

In order to predict the removal and fate of target compounds during activated sludge process, Eqs. (3) and (4) were used (Tchobanoglous et al., 2002) for two full-scale STPs operating at SRTs of 18 d and 8 d (Table S3):

$$M_{in} = M_{bio\text{-}anox} + M_{bio\text{-}aer} + M_{sorbed} + M_{out} \quad (3)$$

where M_{in} and M_{out} are the masses of target compounds in influent and effluent wastewater, respectively (mg d^{-1}), $M_{bio-anox}$ and $M_{bio-aer}$ are the masses of target compounds that are biodegraded in the anoxic and the aerobic bioreactor, respectively (mg d^{-1}) and M_{sorbed} the mass of each target compound removed with excess sludge from the bioreactors (mg d^{-1}).

$$C_{in}Q_{in} = (k_{bio\text{-}anox}C_{out}XV_{anox}) + (k_{bio\text{-}aer}C_{out}XV_{aer}) + \left(\frac{XVK_dC_{out}}{SRT}\right) + (Q_{out}C_{out}) \quad (4)$$

where C_{in} and C_{out} are the concentrations of target compounds in influent and effluent wastewater, respectively (mg m^{-3}), Q_{in} and Q_{out} are the flow rates in influent and effluent wastewater,

respectively ($m^3\ d^{-1}$), $k_{bio-anox}$ and $k_{bio-aer}$ are the experimentally calculated normalized biodegradation constants under anoxic and aerobic conditions, respectively ($L\ g_{MLSS}^{-1}\ d^{-1}$), X is the concentration of MLSS in full-scale bioreactors ($g_{MLSS}\ L^{-1}$), V_{anox} and V_{aer} are the volumes of anoxic and aerobic full-scale bioreactors, respectively (m^3), K_d is the experimentally calculated sludge-water distribution coefficient ($L\ g^{-1}$) and SRT is the sludge residence time in activated sludge bioreactors (d).

The software GraphPad Prism 5 for Windows was used for data evaluation.

3. Results and discussion

3.1. Sorption experiments

The sorption of target compounds was studied in parallel batch reactors, containing different concentrations of the investigated compounds. In order to calculate sorption constants of BTRs and OHBTH, the concentration of each compound in the particulate phase (q_e) was plotted with the concentration of each compound in the dissolved phase (C_e), after 24 h of reaction. Sorption constants varied from 87 to 220 $L\ kg^{-1}$ (Table 2), proving the general hypothesis that the compounds have a weak tendency to sorb onto organic matter. These results are in accordance with a previous study that calculated sorption constants of BTR, and OHBTH using full-scale monitoring data of a STP (Stasinakis et al., 2013). To the best of our knowledge, so far, no data is available in the literature for the sorption of other BTRs.

3.2. Biodegradation experiments

Different batch experiments were conducted using activated and sterilized sludge to study biodegradation potential of BTRs and OHBTH during wastewater treatment and to investigate the role of different factors on their elimination. Monitoring of the total (dissolved + particulate) concentrations of target compounds in abiotic experiments showed no removal of these compounds due to abiotic causes. On the other hand, in the presence of activated sludge, the total concentration of four (BTR, CBTR, XTR and OHBTH) out of six target compounds was significantly decreased up to the end of the experiments (72 h), under both aerobic and anoxic conditions (Fig. S3a, b), indicating that bacteria can biodegrade these compounds using both molecular oxygen and nitrate as electron donors. In all biotic experiments, no significant removal of 4TTR was noticed; whereas removal of 5TTR ranged between 20% and 38% (Fig. S4). Previous studies also reported absence of removal of 4TTR during activated sludge process (Weiss et al., 2006; Herzog et al., 2014a), while biodegradation of 5TTR seems to be slow (complete removal after 91 d, according to Liu et al., 2011) which is enhanced by adaptation of microorganisms (Herzog et al., 2014b). The experiment on micropollutants' partitioning during biodegradation experiments showed, as expected, that the greatest part of the target compounds were in the dissolved phase and this part significantly decreased during the experiment. As an example, the behavior of BTR in Experiment C is given in Fig. S5.

For the four compounds (BTR, CBTR, XTR and OHBTH), which were removed to an extent higher than 50% during biodegradation experiments, a first order kinetic equation was fitted, taking into concern the three individual experiments conducted for each condition examined (Table 1). The biodegradation rate constant (k) and the half life of each compound were calculated with 95% confidence intervals (Table 3). According to these results, the lowest half-life value ($t_{1/2}$ = 6.5 h) was calculated for OHBTH under aerobic conditions, SRT of 18 d and in the presence of supplementary organic substrate (Experiment C). To the best of our knowledge, no information is available in the literature for the biodegradation kinetics of this compound by activated sludge. Regarding BTR, CBTR and XTR, the lowest estimated half-life values were 23 h (Experiment C: aerobic conditions, SRT 18 d, addition of organic substrate), 18 h (Experiment D: anoxic conditions, SRT 18 d, addition of organic substrate) and 14 h (Experiment C: aerobic conditions, SRT 18 d, addition of organic substrate), respectively. So far, contradictory half-life values have been reported in the literature for BTR, ranging from 1 d (Huntscha et al., 2014) to 49 d (Herzog et al., 2014a); whereas no data is available for XTR.

Table 2
Sludge-water distribution coefficients (K_d) determined in batch experiments with activated sludge. The 95% confidence intervals of the measured K_d values are given in parenthesis.

Compound	K_d ($L\ kg^{-1}$)	R^2
BTR	220 (±9)	0.993
4TTR	170 (±48)	0.870
5TTR	165 (±14)	0.979
CBTR	242 (±5)	0.998
XTR	87 (±17)	0.930
OHBTH	147 (±29)	0.893

Table 3
First order kinetics (k), half-life values and biodegradation constants (k_{bio}) calculated in batch experiments with activated sludge under different experimental conditions. The 95% confidence intervals of measured values are given in parenthesis.

Compound	Experiment	k (d^{-1})	Half life (h)	R^2	k_{bio} ($L\ g_{SS}^{-1}\ d^{-1}$)
BTR	Aerobic, SRT 18 d	0.38 ± 0.13	44 ± 18	0.735	0.22 ± 0.08
	Anoxic, STR 18 d	0.41 ± 0.12	40 ± 12	0.807	0.24 ± 0.07
	Aerobic with substrate, STR 18 d	0.73 ± 0.12	23 ± 4	0.947	0.41 ± 0.07
	Anoxic with substrate, SRT 18 d	0.59 ± 0.12	29 ± 6	0.914	0.33 ± 0.07
	Aerobic, SRT 8 d	0.37 ± 0.14	45 ± 21	0.810	0.21 ± 0.08
CBTR	Aerobic, SRT 18 d	0.54 ± 0.06	31 ± 3.5	0.984	0.33 ± 0.04
	Anoxic, STR 18 d	0.75 ± 0.18	22 ± 5.7	0.886	0.45 ± 0.11
	Aerobic with substrate, STR 18 d	0.83 ± 0.24	20 ± 6.4	0.855	0.49 ± 0.14
	Anoxic with substrate, SRT 18 d	0.90 ± 0.25	18 ± 5.5	0.869	0.54 ± 0.15
	Aerobic, SRT 8 d	0.36 ± 0.06	47 ± 17	0.972	0.21 ± 0.04
XTR	Aerobic, SRT 18 d	0.86 ± 0.35	20 ± 9.5	0.759	0.39 ± 0.16
	Anoxic, STR 18 d	0.88 ± 0.26	19 ± 6.0	0.865	0.40 ± 0.12
	Aerobic with substrate, STR 18 d	1.19 ± 0.54	14 ± 8.0	0.759	0.52 ± 0.24
	Anoxic with substrate, SRT 18 d	0.79 ± 0.29	21 ± 8.8	0.801	0.35 ± 0.13
	Aerobic, SRT 8 d	0.64 ± 0.30	26 ± 16	0.790	0.29 ± 0.14
OHBTH	Aerobic, SRT 18 d	0.77 ± 0.34	22 ± 12	0.712	0.40 ± 0.17
	Anoxic, STR 18 d	1.23 ± 0.43	14 ± 5.5	0.849	0.63 ± 0.22
	Aerobic with substrate, STR 18 d	2.58 ± 0.72	6.5 ± 1.9	0.937	1.29 ± 0.36
	Anoxic with substrate, SRT 18 d	1.48 ± 0.33	11 ± 2.6	0.943	0.74 ± 0.16
	Aerobic, SRT 8 d	0.71 ± 0.34	24 ± 15	0.783	0.36 ± 0.18

Concerning the effect of different factors on biodegradation kinetics of the investigated compounds, except for CBTR that was biodegraded faster when biomass with higher SRT was used, no effect was observed for the other compounds (Fig. 1, Table 3: Experiments A, E), indicating that (a) microorganisms capable of degrading these compounds are present in both activated sludge systems, independently of the SRT used and (b) slow-growing bacteria were not playing a significant role on biodegradation of target compounds. These results indicate that biodegradation of these compounds can be expected in all nitrifying conventional and extended aeration activated sludge systems. Further experiments should be conducted at lower SRTs (<8 d) in order to investigate the existence of a critical SRT below which BTRs and OHBTH biodegradation might not occur. Comparison of Experiments A and B showed that the existence of anoxic conditions accelerated biodegradation of CBTR and OHBTH, while no significant difference was observed for the other compounds (Fig. 2, Table 3). No competitive substrate inhibition or catabolic repression of target compounds biodegradation was noticed in the experiments with easily degradable organic substrate (Experiments C, D). On the contrary, the addition of organic substrate resulted in decreased half-life values of BTR (under aerobic and anoxic conditions), CBTR (under aerobic conditions) and OHBTH (under aerobic conditions). It should be mentioned that this acceleration of target compounds biodegradation cannot be explained by the slight biomass increase in experiments with additional organic substrate, as for these compounds a similar trend was also observed in the TSS normalized biodegradation rate coefficients k_{bio} (Table 3). Based on the above, it seems that the increase of biomass in organic-substrate amended batch reactors consisted of bacteria that could also degrade the investigated compounds. Having in mind that (a) the low concentrations of micropollutants added in these experiments ($\mu g\ L^{-1}$) cannot support an significant growth of specified degrading bacteria and (b) no lag phase was noticed in degradation experiments (Fig. 2); it therefore seems that biodegradation of target compounds occurs due to co-metabolic phenomena by microorganisms utilizing a wide range of carbon sources. The aerobic co-metabolic biotransformation of BTR due to hydroxylation of the aromatic benzene ring and methylation of the triazole ring was recently shown by Huntscha et al. (2014).

3.3. Fate during activated sludge process

The calculated sorption and biodegradation constants were used to predict the contribution of different removal mechanisms for BTR, CBTR, XTR and OHBTH during treatment in the activated sludge process system. For this, Eq. (4) was applied for two STPs, operating at SRT of 8 and 18 d (Table S3). According to the model's estimations, all target compounds are partially removed during the activated sludge process, while slightly higher removal efficiency is expected to occur in the STP operating at higher SRT, ranging from 29% for BTR to 46% for OHBTH (Table S4). The partial removal of BTR, CBTR, XTR and OHBTH has also been reported in studies of full-scale STPs (Weiss et al., 2006; Liu et al., 2012; Stasinakis et al., 2013). Due to the low K_d constants of the investigated compounds, the contribution of sorption were of minor importance for their removal and varied from 0.5% (XTR, SRT: 18 d) to 2.7% (CBTR, STR: 8 d) (Table S4). Comparing different STPs, higher removal due to sorption was observed in the case of lower SRT and this is due to the higher production and removal of excess sludge under these conditions. Specifically, excess sludge production was 149 g m^3 in STP with SRT 8 d, comparing to 92 g m^3 at STP with SRT 18 d (Table S3). On the other hand, biotransformation in aerobic and anoxic bioreactors seems to be the major mechanism for their removal, ranging from 22% (BTR, SRT: 8 d) to 45% (OHBTH, STR: 18 d) (Table S4). As aerobic biodegradation constants ($k_{bio\text{-}aer}$) were similar or smaller than those calculated under anoxic conditions ($k_{bio\text{-}anox}$) for all target compounds (Table 3); the higher contribution of aerobic bioreactor on their removal is mainly due to the greater volume of this reactor and to the relative greater mass of involved microorganisms comparing to the anoxic.

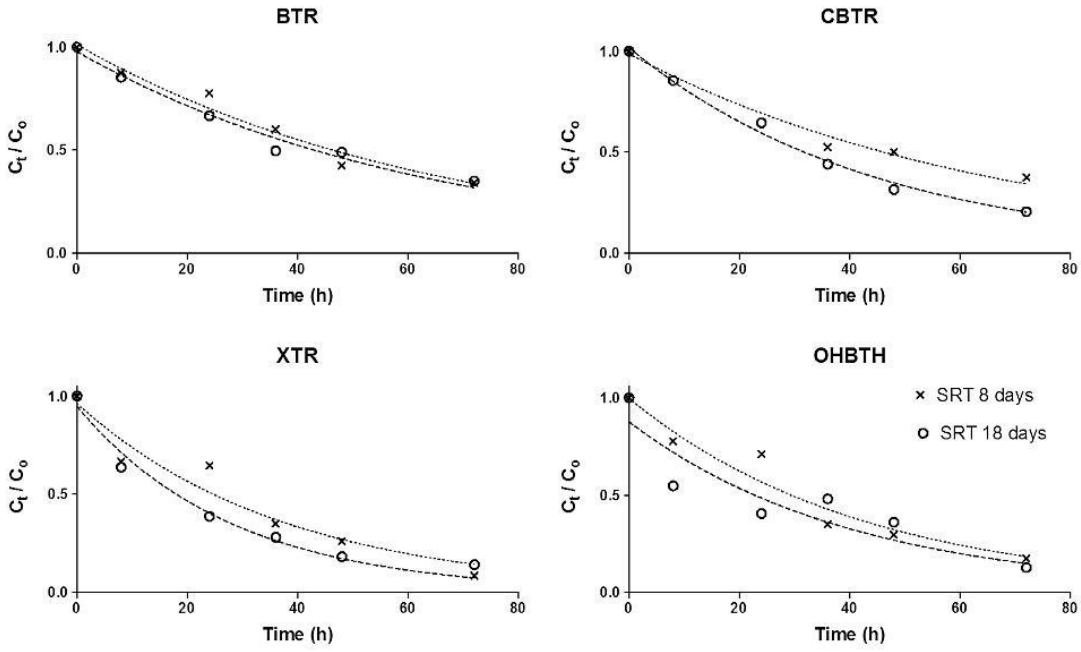

Fig. 1. Effect of SRT on changes of total (dissolved + particulate) relative concentrations of BTR, CBTR, XTR and OHBTH.

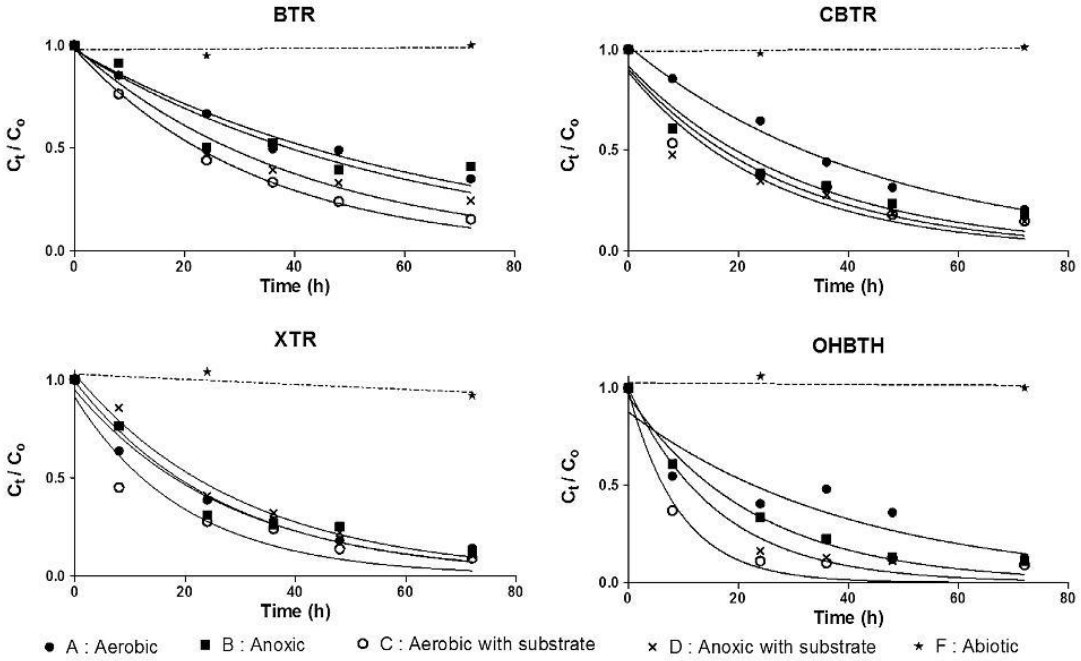

Fig. 2. Changes of total (dissolved + particulate) relative concentrations of BTR, CBTR, XTR and OHBTH in different biodegradation experiments (A: aerobic, SRT 18 d, B: anoxic, STR 18 d, C: aerobic with addition of substrate, STR 18 d, D: anoxic with addition of substrate, SRT 18 d, F: abiotic).

To investigate model's sensitivity to different factors which could affect the prediction of the removal of the investigated compounds, three different scenarios were tested. Specifically, by increasing MLSS concentration in anoxic and aerobic bioreactors from 3000 mg L^{-1} to 5000 mg L^{-1}, an increase of the total removal efficiency equal to 10–13% was calculated for target compounds (Table S5). For the case that k_{biol} constants were 20% higher than those experimentally calculated, 3% (BTR) to 5% (CBTR) higher removal would be observed in a STP operating at SRT of 18 d (Table S5). Similarly, an overestimation of k_{biol} by 20% would decrease total removal of these compounds from 4% (BTR) to 5% (OHBTH, CBTR, XTR). These results indicate that the operation of full-scale bioreactors at higher MLSS concentrations with constant sludge retention time could improve removal of these compounds, while possible errors on calculation of biodegradation constants affect slightly their predicted elimination rates.

4. Conclusions

BTR, CBTR, XTR, and OHBTH were significantly removed during aerobic and anoxic activated sludge experiments and their biodegradation half-lives varied from 6.5 h (OHBTH) to 47 h (CBTR). The removal of these compounds in STPs does not seem to require special conditions, whereas it is not dramatically enhanced or inhibited by the variation of parameters such as SRT value. On the other hand, 4TTR was not removed, while 5TTR was only partially removed. Biodegradation of target compounds seems to occur due to co-metabolism by microorganisms that use either molecular oxygen or nitrates as electron donors and scavenge a wide range of carbon sources. Partial removal of the investigated compounds is expected during biological wastewater treatment ranging from 29% for BTR to 46% for OHBTH (for a STP operating at SRT of 18 d); the greatest removal is due to biodegradation occurring in aerobic bioreactors, while the role of sorption is minor. The use of consecutive final treatment stages such as ozonation or activated carbon is needed to be evaluated in order to assess the degree of removal of target compounds and to decide whether this could be a treatment option. Furthermore, the investigation of possibly formed transformation products has to be included in future studies.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2015.03.029.

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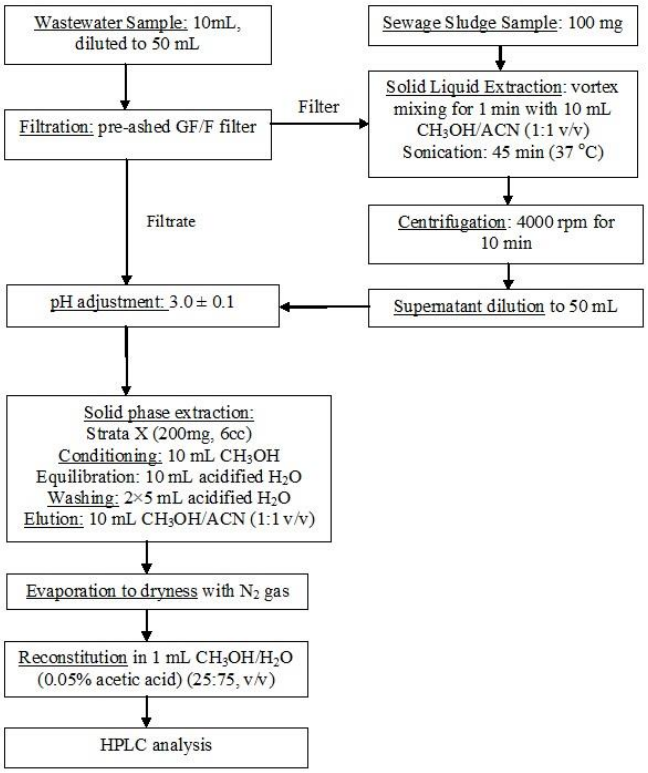

Figure S1. Schematic diagram of the applied analytical method for the determination of the target BTRs and OHBTH in wastewater and sludge samples (Asimakopoulos et al., 2013).

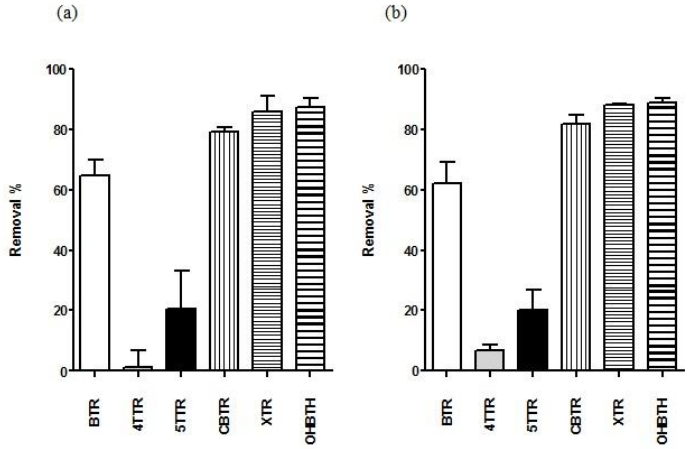

Figure S2 Removal efficiency (%) of BTRs and OHBTH in biodegradation batch experiments conducted under a) aerobic conditions (Experiment A, 72 h), b) anoxic conditions (Experiment B, 72 h)

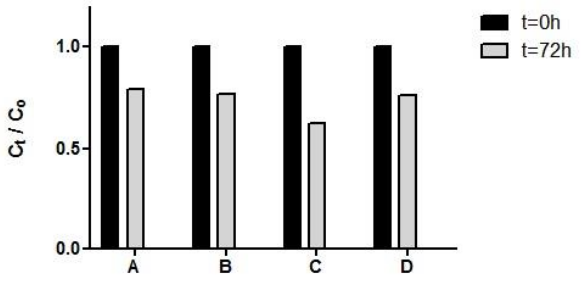

Figure S3. Initial (t = 0h) and final (t = 72h) concentration (C_t/C_o) of 5TTR in different biodegradation experiments (A: aerobic, SRT 18d, B: anoxic, STR 18 d C: aerobic with addition of substrate, STR 18 d, D: anoxic with addition of substrate, SRT 18 d)

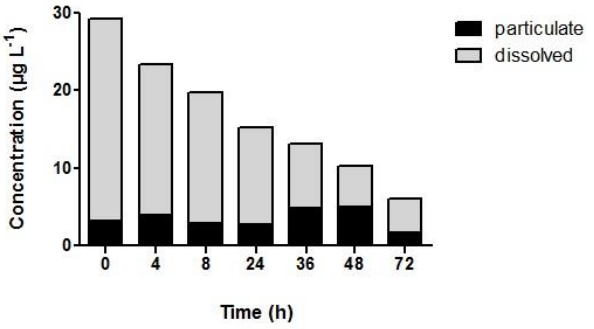

Figure S4. Change of particulate and dissolved BTR concentration under aerobic conditions and presence of organic substrate (Experiment C).

Table S1. Target compounds that were analyzed in the present study.

Compound	Molecular Formula	M.W.	LogK$_{ow}$	pKa
1H-benzotriazole (BTR)	$C_6H_5N_3$	119.12	1.23[2]	8.37[1]
4-Methyl-1H-benzoriazole (4TTR)	$C_7H_7N_3$	133.15	1.89[2]	8.5[2]
5-Methyl-1H-benzoriazole (5TTR)	$C_7H_7N_3$	133.15	1.89[2]	8.5[2]
5,6-dimethyl-1H-benzotriazole or xylytriazole (5,6 DMTR or XTR)	$C_8H_9N_3$	147.18	2.06[5]	9.28[5]
5-Chlorobenzotriazole (CBTR)	$C_6H_4ClN_3$	153.57	2.17[6]	7.5/7.7[6]
2-hydroxybenzothiazole (OHBTH)	C_7H_5NSO	151.2	1.76[3]	8.65[4]

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Table S2. Precision, trueness and limits of detection (LODs) and quantification (LOQs) of the analytical methods.

Compound	Intra-day precision (RSD %, n = 6)	Inter-day precision (RSD %, n = 3)	Trueness (Recovery, %) n = 4	LOD (ng L^{-1} in sample)	LOQ (ng L^{-1} in sample)
Dissolved phase					
BTR	8.9	1.1	73.9 - 82.9	17	52
4TTR	12.0	9.6	36.1 - 54.8	28	84
5TTR	7.7	7.4	60.6 - 68.8	23	69
CBTR	9.3	6.7	72.7 - 82.0	125	376
XTR	9.7	10.1	60.1 - 85.0	107	322
OHBTH	10.4	10.9	69.8 – 73.0	30	90
Compound	**Intra-day precision (RSD %, n = 6)**	**Inter-day precision (RSD %, n = 3)**	**Trueness (Recovery, %) n = 4**	**LOD (ng g^{-1} in sample)**	**LOQ (ng g^{-1} in sample)**
Particulate phase					
BTR	10.8	7.4	59.8 - 60.8	40	118
4TTR	10.5	6.6	53.6 - 77.5	368	1104
5TTR	11.0	11.6	67.1 - 73.5	555	1666
CBTR	13.8	14.0	64.8 - 69.1	132	397
XTR	11.1	11.3	50.8 - 54.0	236	709
OHBTH	6.5	5.9	70.1 - 74.8	72	216

Table S3. Data used in model application for estimating BTRs and OHBTH fate during activated sludge process.

Parameters	STP A (Mytilene, Greece)	STP B (Athens, Greece)
Q_{in} (m^3 d^{-1})	7400	710000
Q_{out} (m^3 d^{-1})	7285	670000
Volume of anoxic bioreactor (m^3)	1370	94560
Volume of aerobic bioreactor (m^3)	2730	186792
SRT (d)	18	8
HRT (h)	24	10
MLSS in activated sludge bioreactors (mg L^{-1})	3000	3000
$P_{X,MLSS}$ (kg d^{-1})	683	105507
Excess sludge production (g m^{-3})	92	149

Table S4. Contribution of different mechanisms to the removal of the investigated compounds during activated sludge treatment in typical STPs operating either at SRT of 8 d or 18 d. Predictions are based on the experimentally determined sorption and biodegradation constants.

Compound	Removal (%) in a STP operating at a SRT of 18 d				Removal (%) in a STP operating at a SRT of 8 d			
	Anoxic biodegradation	Aerobic biodegradation	Sorption	Total	Anoxic biodegradation	Aerobic biodegradation	Sorption	Total
BTR	9.7	18	1.5	29	7.8	14	2.6	24
CBTR	16	22	1.4	39	14	13	2.7	30
XTR	14	26	0.5	41	12	17	1.0	30
OHBTH	20	25	0.8	46	17	19	1.5	38

Table S5. Model's sensitivity concerning the total removal of target compounds during activated sludge process in typical STPs operating at SRT of 8 d and 18 d (A: prediction based on the experimentally determined biodegradation constants and MLSS concentration of 3000 mg L^{-1}. B: prediction based on the experimentally determined biodegradation constants and MLSS concentration of 5000 mg L^{-1}. C: prediction based on biodegradation constants higher by 20% comparing to those experimentally determined and MLSS concentration of 3000 mg L^{-1}. D: prediction based on biodegradation constants lower by 20% comparing to those experimentally determined and MLSS concentration of 3000 mg L^{-1}).

Compound	Total Removal (%) in a STP operating at a SRT of 18 d				Total Removal (%) in a STP operating at a SRT of 8 d			
	A	B	C	D	A	B	C	D
BTR	29	40	32	25	24	34	27	21
CBTR	39	52	44	34	29	41	33	25
XTR	40	53	44	35	30	41	34	25
OHBTH	45	58	49	40	37	50	41	32

Paper B

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Biodegradation of benzotriazoles and hydroxy-benzothiazole in wastewater by activated sludge and moving bed biofilm reactor systems

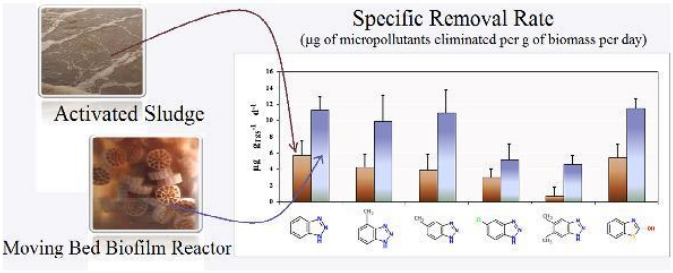
Aikaterini A. Mazioti [a], Athanasios S. Stasinakis [a,b,*], Ypapanti Pantazi [a], Henrik R. Andersen [b]

[a] Department of Environment, University of the Aegean, Mytilene, Greece
[b] Department of Environmental Engineering, Technical University of Denmark, Miljøvej, B 113, 2800 Kgs. Lyngby, Denmark

HIGHLIGHTS

- All target compounds were biodegradable in both activated sludge and MBBR systems.
- Operation of MBBR at low organic loading enhanced micropollutants removal.
- Attached biomass exhibited greater ability to remove micropollutants.
- Biodegradation potential in biofilm differed in each bioreactor of MBBR system.
- Biodegradation of micropollutants occurs due to co-metabolic phenomena.

GRAPHICAL ABSTRACT



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ABSTRACT

Two laboratory scale fully aerated continuous flow wastewater treatment systems were used to compare the removal of five benzotriazoles and one benzothiazole by suspended and attached growth biomass. The activated sludge system was operated under low organic loading conditions. The moving bed biofilm reactor (MBBR) system consisted of two serially connected reactors filled with K3-biocarriers. It was either operated under low or high organic loading conditions. Target compounds were removed partially and with different rates in tested systems. For MBBR, increased loading resulted in significantly lower biodegradation for 4 out of 6 examined compounds. Calculation of specific removal rates (normalized to biomass) revealed that attached biomass had higher biodegradation potential for target compounds comparing to suspended biomass. Clear differences in the biodegradation ability of attached biomass grown in different bioreactors of MBBR systems were also observed. Batch experiments showed that micropollutants biodegradation by both types of biomass is co-metabolic.

1. Introduction

Benzotriazoles (BTRs) and benzothiazoles (BTHs) are two classes of polar emerging contaminants that are frequently detected in surface water all over the world (Ni et al., 2008; Loos et al., 2009; Nödler et al., 2014). Due to their widespread use in several industrial applications and everyday products (anticorrosive and antifreezing products, drugs, ultraviolet stabilizers), sewage is considered their main pathway to the aquatic environment and concentrations up to some $\mu g\,L^{-1}$ are frequently detected (Liu et al., 2012; Thomaidis et al., 2012; Stasinakis et al., 2013).

According to monitoring studies conducted in full-scale Sewage Treatment Plants (STPs), BTRs and BTHs are partially removed during conventional wastewater treatment (Reemtsma et al., 2010;

* Corresponding author at: Department of Environment, University of the Aegean, Mytilene, Greece. Tel.: +30 22510 36257; fax: +30 22510 36206.
E-mail address: astas@env.aegean.gr (A.S. Stasinakis).

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Stasinakis et al., 2013). Some recent studies have focused on the aerobic biodegradation rates of these compounds in activated sludge (AS) batch experiments, while continuous-flow experiments have so far not been performed to investigate their removal in AS systems. Specifically, Liu et al. (2011) investigated biodegradation of benzotriazole (BTR), 5-methy-1H-lbenzotriazole (5TTR) and 5-chlorobenzotriazole (CBTR) in batch experiments conducted under different conditions and calculated their kinetic parameters, while Huntscha et al. (2014) calculated half-lives and biotransformation products of selected BTRs. Herzog et al. (2014a,b) studied the removal efficiency of BTR, 4-methyl-1H-benzotriazole (4TTR) and 5TTR under different experimental conditions and reported that sludge acclimatization enhanced biodegradation of some compounds. In a recent study focusing on biodegradation and sorption, Mazioti et al. (2015) reported that BTR, CBTR, xylytriazole (XTR), and 2-hydroxybenzothiazole (OHBTH) can be biodegraded by AS with half-lives varying from 6.5 h to 47 h, while sorption contributes weakly to their elimination.

Concerning biological wastewater treatment, a novel type of treatment has been developed by the late 1980s in Norway (Barwal and Chaudhary, 2014). This type of treatment profited of the microorganisms trend to form biofilms and is nowadays giving a promising option for wastewater treatment. Moving bed biofilm reactor (MBBR) systems have been used both in pilot plant studies and in full scale plants for the treatment of different types of wastewater (Barwal and Chaudhary, 2014). MBBRs are usually filled with plastic biocarriers, on which biomass is attached, and circulate in all parts of the reactor with the aid of aeration or mechanical stirring. Some of the attached biomass advantages are their ability to cope with high loading conditions, the capacity of treatment of both industrial and municipal wastewater at a relatively low footprint and the avoidance of excess sludge removal (Loupasaki and Diamadopoulos, 2013; Shahot et al., 2014). Due to these advantages attached biomass presents, many previous studies have focused on the operation of MBBRs pilot systems investigating the removal of conventional pollutants from sewage (Di Trapani et al., 2011; Ibrahim et al., 2014; Zhang et al., 2014 and Gilbert et al., 2014). On the other hand, though biofilms may be a key technology for the removal of toxic and emerging pollutants (Borghei and Hosseini, 2004; Edwards and Kjellerup, 2013), only few studies have examined the removal of micropollutants using MBBRs. Specifically, Falås et al. (2012) investigated pharmaceuticals degradation and calculated removal rate constants in batch experiments with carriers that had been collected from different full-scale STPs, while in a recent work they investigated the removal of 20 micropollutants by monitoring a full scale hybrid biofilm/AS plant (Falås et al., 2013). In another study, the removal of three hormones was examined by early-stage biofilm in batch tests (Khan et al., 2013). Luo et al. (2014) operated a bench-scale MBBR system with polyurethane sponge carriers in order to determine various micropollutants removal. Finally, Accinelli et al. (2012) examined the removal of bisphenol-A, atrazine and oseltamivir with bioplastic carriers inoculated with specific bacterial strains. Concerning BTRs and BTHs, so far their removal in MBBR systems has not been studied and their biodegradation constants have not been calculated using attached biomass. Moreover, to the best of our knowledge, no studies are available comparing the ability of suspended and attached biomass to remove micropollutants. Limited information is also available for the role of organic loading (Ahmadi et al., 2015) and the contribution of different reactors in series on micropollutants removal in a MBBR system.

Based on the above, the main objective of this study was to examine two different types of biological treatment (AS and MBBR) in order to compare their ability to remove BTR, CBTR, XTR, 4TTR, 5TTR and OHBTH from domestic wastewater. For this reason, two continuous-flow laboratory scale systems were installed and operated under different hydraulic retention time (HRT). Both systems were monitored during adequate period of time for the elimination of conventional wastewater parameters and target micropollutants and the specific removal rates (as μg of compound per gram of biomass and day) were calculated for each target micropollutant. Special focus was given on the contribution of different bioreactors of MBBR system on the removal of micropollutants and on the biodegradation potential of developed biomass in different bioreactors. Batch experiments were also conducted using AS and biomass from MBBR systems in order to determine the role of organic substrate, measured as chemical oxygen demand (COD) on biodegradation kinetics. The calculated biodegradation constants were used in order to predict the removal of target compounds in applied systems and consequently evaluate their accuracy.

2. Methods

2.1. Analytical standards and reagents

Analytical standards of XTR and CBTR were supplied by Sigma-Aldrich (USA). BTR was purchased from Merck (Germany), 4TTR by Fluka (Switzerland), 5TTR by Acros Organics (Belgium); whereas OHBTH was purchased from Alfa Aesar (USA). Stock solutions of individual compounds were prepared in methanol (MeOH) at 1000 mg L^{-1} and kept at −18 °C. Working solutions of 10 mg L^{-1} were prepared when needed and were kept at −18 °C for a time period not exceeding three months. Methanol (MeOH; HPLC-MS grade) and acetonitrile (ACN; HPLC grade) were purchased from Merck (Germany) and Fisher (USA), respectively. The solid phase extraction (SPE) cartridges used for samples' clean-up were polymer-based with surface modified styrene divinylbenzene phase (Strata-X, 33u Polymeric Reversed Phase 200 mg/6 ml) and they were supplied by Phenomenex (USA). HPLC grade water was prepared in the laboratory using a MilliQ/MilliRO Millipore system (USA). Ultra-pure HCl (32%), used for samples acidification, was purchased from Merck (Germany).

2.2. Continuous flow systems: set-up and operation

Small scale continuous flow systems were installed and operated in the laboratory (Fig. S1), under constant room temperature controlled by central air-conditioning system. The AS system consisted of an aerobic bioreactor (AB), with a working volume of 4.5 L, and a settling tank with a working volume of 1 L, from which sludge was recirculating to the bioreactor (Solid Retention Time, SRT: 18 d; HRT: 26.4 ± 2.4 h; organic loading: 0.25 ± 0.16 kg m^{-3} d^{-1}). The AS for AB start-up was taken from a nitrifying municipal STP (Mytilene, Greece), operating at a SRT of 18 d; the laboratory scale system operated in summer 2014. The MBBR system consisted of two aerobic bioreactors (BC1 and BC2) connected in series, with a working volume of 4.5 L each. Each bioreactor contained biocarriers (type K3, AnoxKaldnes) at a filling ratio of 30%. The biocarriers were moving due to aeration in all parts of the reactor. The MBBR system was operated at two HRTs, in two different experimental cycles during summer and autumn 2014. A HRT of 26.4 ± 3.6 h (for each reactor) was applied in the first experimental cycle, providing a low substrate organic loading (MBBR-low) equal to 0.25 ± 0.16 kg m^{-3} d^{-1} for BC1 and 0.05 ± 0.03 kg m^{-3} d^{-1} for BC2. A lower HRT of 10.8 ± 1.2 h (for each reactor) was applied in the second experimental cycle in order to provide higher substrate organic loading (MBBR-high), equal to 0.60 ± 0.40 kg m^{-3} d^{-1} for BC1 and 0.17 ± 0.11 kg m^{-3} d^{-1} for BC2. All systems were fed with raw wastewater collected from

the STP of the University Campus in Mytilene, Greece (Table S1). In all bioreactors, the conservation of aerobic conditions and adequate mixing of biomass were achieved by using porous ceramic diffusers, while dissolved oxygen concentration (DO) was higher than 4 mg L^{-1}. In order to develop a stable biofilm onto the carriers, the MBBR system was operated for 5 months with domestic wastewater before starting the experiments with micropollutants.

An acclimatization phase of 3–4 weeks took place for both systems, during which conventional pollutants removal was frequently examined in order to ensure AS and MBBRs stability and efficient performance. After this period of time, the target compounds were spiked using methanol solutions, in order to obtain a daily stable concentration inflow of approximately 20 μg L^{-1}. In order to evaluate the removal of target compounds, samples were taken for 10 consecutive days from different sampling points of the systems (Fig. S1). To control the operation of continuous-flow systems, COD, NH_4-N, NO_3-N, mixed liquor suspended solids (MLSS), total suspended solids (TSS), attached biomass onto biocarriers, temperature (T), DO and pH were measured at predetermined time intervals.

2.3. Batch biodegradation experiments

To calculate biodegradation kinetics of target compounds and to investigate the effect of organic substrate availability on their biodegradation, batch experiments were also conducted with both types of biomass. AS was collected from a nitrifying STP (Mytilene, Greece) during autumn 2014, while attached biomass was used from the second MBBR experimental cycle (MBBR-high) that was running that period. AS experiments were conducted in stoppered glass bottles that were constantly agitated on a shaking plate. The working volume in each reactor was 1 L and the mixed liquor suspended solids (MLSS) concentration was equal to 3000 ± 200 mg L^{-1}. Experiments with attached biomass were conducted in the bioreactors (BC1, BC2) by stopping the flow and operating them under batch conditions for 24 h. The investigated compounds were spiked using methanolic solutions to obtain an initial concentration of approximately 30 μg L^{-1} for each micro-contaminant in the reactors. Two different COD concentrations were tested (30 mg L^{-1} and 270 mg L^{-1}), corresponding to a low and moderate organic loading of 0.03 kg m^{-3} d^{-1} and 0.27 kg m^{-3} d^{-1}, respectively. To quantify biodegradation of micropollutants, homogenized samples (50 mL) were collected after 0, 1, 2.5, 5, 12 and 24 h. Since sorption to organic matter is of minor importance for these groups of compounds (Mazioti et al., 2015), the concentrations of target compounds were determined only in the dissolved phase using the analytical method described below. All batch experiments were conducted at 22.0 ± 0.5 °C, pH ranged between 6.3 and 7.4, while DO was higher than 4 mg L^{-1}.

2.4. Analytical methods

Analysis of COD, NH_4-N, NO_3-N, TSS and MLSS were performed according to Standard Methods (APHA, 1998), T, DO and pH were measured using portable instruments. The quantification of the attached biomass occurred by removing the biofilm from biocarriers and measuring the dried weight difference, as described by Falås et al., 2012. Microscopic observations were also conducted in order to check AS process (Jenkins et al., 2003) and biofilm formation.

For the investigation of target compounds fate, samples were filtered through glass fiber filters (GF-3 Macherey Nagel). Filtrates were collected, acidified to pH 3.0 ± 0.1 and stored at 4 °C until analysis. Analysis of target compounds in the dissolved phase was based on previously developed methods (Asimakopoulos et al., 2013; Mazioti et al., 2015) and included solid phase extraction (SPE). Chromatographic analysis was performed by a Shimatzu (Japan) LC20-AD prominence liquid chromatographer associated with a SPD-M20A prominence diode array detector and a SIL-20AC auto sampler. Satisfactory recoveries and precision of the analytical procedure was achieved; whereas the obtained LODs ranged from 17 ng L^{-1} (BTR) to 125 ng L^{-1} (CBTR). Further information for the analytical method and the chromatographic conditions can be found in a recently published study (Mazioti et al., 2015).

2.5. Equations

Micropollutants removal in laboratory scale reactors was calculated according to Eq. (1):

$$\text{Removal} = \left(1 - \frac{C_{out}}{C_{in}}\right) \times 100 \quad (1)$$

where C_{in} is the concentration of target compound in influent wastewater (μg L^{-1}) and C_{out} the concentration in treated wastewater of each examined reactor (μg L^{-1}).

Specific removal rate for each compound and type of biomass was calculated according to Eq. (2):

$$\text{Specific removal rate} = \left(\frac{C_{in}Q_{in} - C_{out}Q_{out}}{X \times V}\right) \quad (2)$$

where Q_{in} and Q_{out} the flow rates of influent and effluent wastewater, respectively (L d^{-1}), X the concentration of attached or suspended biomass (g L^{-1}) and V the volume of each bioreactor (L).

The biodegradation rate constants (k) were estimated using first order kinetics. Pseudo first-order biodegradation rate coefficient, k_{bio}, normalized to attached or suspended biomass (L g_{MLSS}^{-1} d^{-1}) was calculated for each biodegradation experiment using the appropriate sorption constant (K_d as L g^{-1}) for each compound (Mazioti et al., 2015) and Eq. (3) (Ziels et al., 2014):

$$\ln\frac{C_t}{C_0} = -k_{bio} \times \left(\frac{X}{1 + K_d X}\right) \times t \quad (3)$$

where C_t and C_0 are the dissolved target compound concentrations in batch experiment at time t and $t = 0$, respectively, (μg L^{-1}).

Predicted removal in continuous-flow systems was estimated using the first-order biodegradation rate constants (k) calculated in batch experiments, according to equation was (4):

$$\text{Predicted removal} = 1 - \left(\frac{1}{(1 + k_1\tau_1)(1 + k_2\tau_2)}\right) \quad (4)$$

where τ is the hydraulic retention time for each reactor; in the case of the MBBR system (τ_1, τ_2), while for the AS system only one reactor was used (τ_1).

2.6. Statistical analysis

In order to compare the removal values and specific removal rates one-way ANOVA was used with Tukey–Kramer's post-test for significant differences between groups.

3. Results and discussion

3.1. Operation of continuous-flow AS and MBBR systems

The operational parameters of the continuous flow systems are presented in Table 1. Both systems adequately eliminated organic loading from wastewater, achieving similar average dissolved COD removal equal to 86% (MBBR-low) and 90% (AS). Both systems were also able to remove NH_4-N sufficiently (average removal 93–95%). During microscopic observations, protozoa, rotifers and filamentous

Table 1
Operational parameters of continuous flow systems, during acclimatization and loading with target compounds: activated sludge (AS, HRT 26.4 ± 2.4 h), Biocarriers under low loading conditions (MBBR-low, HRT 26.4 ± 3.6 h for each reactor) and biocarriers under high loading conditions (MBBR-high, HRT 10.8 ± 1.2 h for each reactor).

Activated sludge system														
Continuous flow system	Days of operation			MLSS (mg L^{-1})	TSS (mg L^{-1})	pH		Removal %						
								COD dissolved			NH_4-N			
				AB[a]	Out[b]	AB[a]	Out[b]	AB			AB			
AS (n = 16)	31			2370 (±590)	11 (±13)	7.2 (±0.4)	7.3 (±0.6)	90 (±7)			93 (±12)			
Moving bed bioreactor system														
Continuous flow system	Days of operation	Attached Biomass (mg L^{-1})		MLSS (mg L^{-1})	MLSS (mg L^{-1})	pH		Removal %						
								COD dissolved			NH_4-N			
		BC1[c]	BC2[d]	BC1[c]	BC2[d]	BC1[c]	BC2[d]	BC1[c]	BC2[d]	Total[e]	BC1[c]	BC2[d]	Total[e]	
MBBR-low (n = 15)	45	726[f]	100[f]	195 (±81)	131 (±89)	7.0 (±0.5)	6.8 (±0.9)	81 (±13)	42 (±26)	86 (±11)	78 (±29)	84 (±23)	93 (±13)	
MBBR-high (n = 11)	45	1079[g] (±715)	312[g] (±108)	138 (±68)	124 (±68)	7.4 (±0.2)	7.2 (±0.3)	72 (±11)	67 (±21)	91 (±7)	73 (±24)	87 (±21)	95 (±7)	

[a] AB: aerobic bioreactor with activated sludge.
[b] Out: treated wastewater.
[c] BC1: bioreactor with biocarriers 1.
[d] BC2: bioreactor with biocarriers 2.
[e] Total: total removal in BC1 and BC2.
[f] Attached biomass in MBBR-low was measured once.
[g] Attached biomass in MBBR-high was measured thrice.

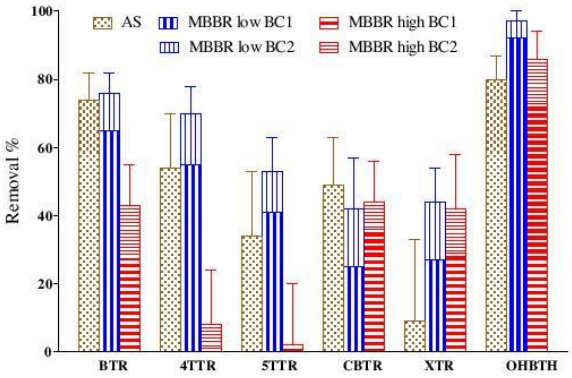

Fig. 1. Removal (%) of target compounds in activated sludge (AS) and moving bed biofilm reactor (MBBR) system operated under low (MBBR-low) and high organic loading (MBBR-high) conditions (t-bars represent 95% confidence interval). The contribution of each bioreactor (BC1 and BC2) to target compounds removal is also shown.

bacteria were identified in the AS system, indicating a stable and mature environment (Jenkins et al., 2003). Metazoa and protozoa were also observed in the MBBR system (Fig. S2). In the AS system, MLSS decreased slightly during the acclimatization phase, but the concentration remained stable (2230 ± 290 mg L^{-1}) during the experiment with micropollutants. In the MBBR system, a thicker biofilm and a higher concentration of biomass developed in the first bioreactor, BC1 (Table 1 and Fig. S3), probably due to the higher COD concentrations in BC1 comparing to BC2, where most organic substrate had already been consumed. Despite the thinner biofilm in BC2, the developed biomass had a greater ability to nitrify. In the first experimental cycle (low loaded MBBR), on average 170 mg of NH_4-N were removed per day and per gram of biomass in BC1, while 250 mg d^{-1} g^{-1} were removed in BC2. A similar trend was also observed during the second experimental cycle (high loaded MBBR), with nitrification rates being even higher in both reactors (on average 295 mg d^{-1} g^{-1} in BC1 and 480 mg d^{-1} g^{-1} in BC2). Furthermore, in the high loaded MBBR system, a thicker biofilm was observed in both bioreactors.

3.2. Removal of target compounds in continuous-flow systems

The observed removal of target compounds in each system was mainly due to biodegradation as the compounds are known not to be degraded due to abiotic mechanisms, under the conditions found in bioreactors, and they are poorly sorbed onto biomass (Mazioti et al., 2015). Their average removal varied from 43% to 76% for BTR, 8% to 69% for 4TTR, 0% to 53% for 5TTR, 42% to 49% for CBTR, 9% to 43% for XTR and 80% to 97% for OHBTH (Fig. 1), indicating that none of the compounds was totally eliminated during wastewater treatment. Except for CBTR that was removed at the same rate regardless of the treatment type, all other compounds were eliminated to a different degree, depending on the system used.

Table 2
Mass of micropollutants removed per mass of biomass and day during continuous flow experiments with activated sludge (AS), biocarriers under low loading conditions (MBBR-low) and biocarriers under high loading conditions (MBBR-high) (values in bold indicate statistically significant differences).

System	Specific removal (μg of micropollutant removed per g of biomass per day)					
	BTR	4TTR	5TTR	CBTR	XTR	OHBTH
AS	5.7 (±1.8)	**4.2 (±1.6)**	**3.9 (±1.9)**	**3.0 (±1.0)**	**0.7 (±1.1)**	**5.4 (±1.6)**
MBBR-low	**11.3 (±1.6)**	**9.9 (±3.2)**	**10.9 (±2.8)**	5.2 (±1.9)	4.6 (±1.1)	11.5 (±1.2)
MBBR-high	5.7 (±1.9)	**15.1 (±12.3)**	**6.1 (±5.3)**	6.5 (±2.0)	4.4 (±1.9)	11.6 (±2.6)

In order to compare the removal efficiency of a suspended-growth and an attached-growth system operating in parallel under the same organic loading conditions and HRT, AS system and BC1 of MBBR-low system were used. According to Fig. 1, the removal of 4TTR, 5TTR and XTR was similar in both systems, whereas statistically significant differences were observed for BTR (higher in AS), CBTR (higher in AS) and OHBTH (higher in MBBR), indicating that the application of same organic loading and HRT does not necessarily lead to same removal for all micropollutants. The increase of HRT in the low loaded MBBR system via the addition of a second reactor (BC2) enhanced to some degree the removal of micropollutants (up to 15%) but complete removal was

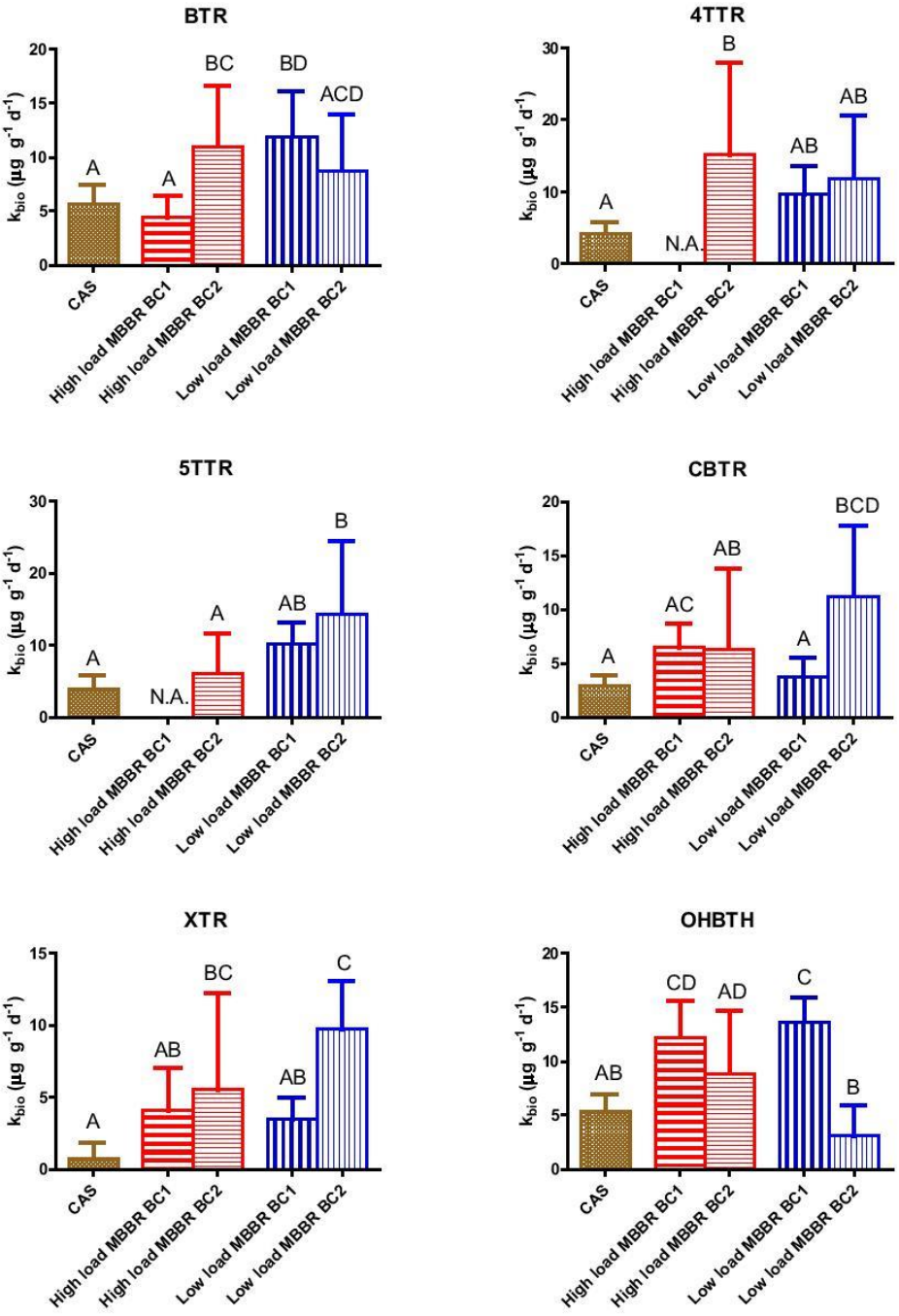

Fig. 2. Mass of micropollutants removed per mass of biomass and day during continuous flow experiments with activated sludge (AS), biocarriers under low loading conditions (MBBR-low) and biocarriers under high loading conditions (MBBR-high). Results are given for each bioreactor (BC1 and BC2), separately (different letters indicate statistical differences at 95% confidence level; t-bars represent 95% confidence interval).

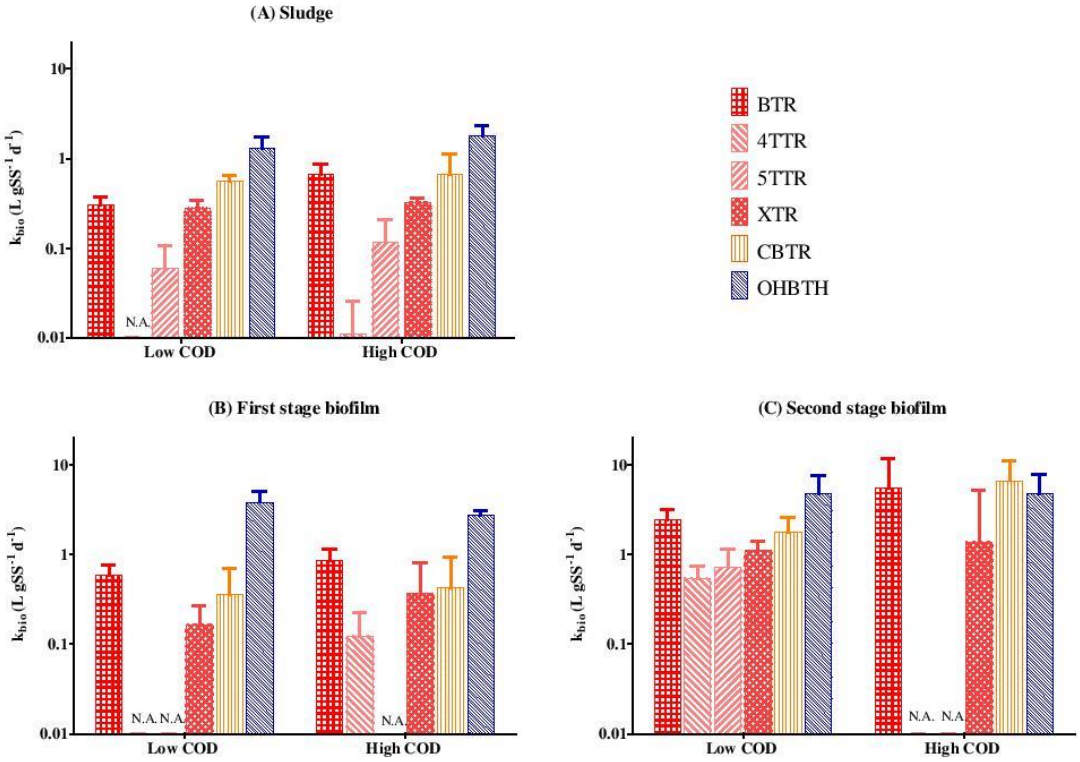

Fig. 3. Effect of low or high COD concentrations on observed biodegradation constants, k_{bio} (L g_{SS}^{-1} d^{-1}) in batch experiments with activated sludge (A) and attached biomass from BC1 (B) and BC2 (C) (t-bars represent 95% confidence interval).

not achieved. Similarly to the current study, Ahmadi et al. (2015) observed a moderate increase of diethylphthalate and diallylphthalate removal when HRT was increased from 3 to 12 h in a MBBR laboratory scale system.

When the MBBR system was operated under a higher organic loading (2nd experimental cycle), the total removal of XTR and CBTR was the same with low loaded MBBR. On the other hand, statistically lower removal was observed for OHBTH, BTR, 4TTR, while 5TTR was not eliminated at all (Fig. 1). Beside the increased biomass developed in high loaded MBBR (Table 1), it seems that the increase of the organic loading in the MBBR system decreased its capacity to remove some of the target compounds. So far, limited results have been published in the literature for the role of organic loading on the removal of micropollutants. Ahmadi et al. (2015) reported that the increase of organic loading from 0.73 to 1.46 kg COD m^{-3} d^{-1} had not actual effect (<1%) on the removal of two phthalic acid esters in a MBBR. To the best of our knowledge, no other studies are available in the literature for the range of organic loadings applied in the current study (0.25 to 0.60 kg m^{-3} d^{-1}) and for the added concentrations of micropollutants (μg L^{-1} levels).

3.3. Ability of different types of biomass to biodegrade target compounds

As the biomass amount was not the same in all bioreactors (Table 1), the specific removal rate (as μg per g and day) was calculated for each micropollutant to compare the ability of biomass developed in each system to remove the target compounds. According to the results presented in Table 2 for total specific removal rate, the attached biomass developed in MBBR systems presented statistically significant higher ability to biodegrade all target compounds comparing to the suspended biomass of AS system. In the low loaded MBBR system, these values ranged between 4.6 (XTR) and 11.3 μg g^{-1} d^{-1} (BTR), while similar (for OHBTH, XTR, CBTR) or lower values (for BTR and 5TTR) were calculated in high loaded MBBR system. This general advantage of the attached biomass over the suspended is probably due to the higher residence time of biomass onto carriers that could allow a richer biodiversity through the protection of slow growing bacteria from washout, which might be capable to remove micropollutants. In a recent study, Zhang et al. (2015) observed significant differences on the microbial communities established in suspended and attached biomass on phylum and genus level. Moreover, Edwards and Kjellerup (2013) reported that a large variety of species of microorganisms is included in biofilms, whereas all of them contribute to each other's metabolic needs.

To investigate whether biomass with the same ability to remove our target compounds is grown in different bioreactors of the MBBR system, specific removal rates were also calculated for BC1 and BC2 of both MBBR systems (Fig. 2 and Table S2). For biomass developed under poor organic loading conditions (MBBR-low), three compounds (BTR, 4TTR and 5TTR) had no different specific removal rate between BC1 and BC2. On the other hand, CBTR and XTR were more efficiently removed in BC2, while for OHBTH the first reactor was more effective. It is worth mentioned that the low removal observed for OHBTH in BC2 could be attributed to the low availability of this compound in this reactor (due to its significant removal in BC1) and not necessarily to the capacity of the biomass. A different trend was observed with biomass

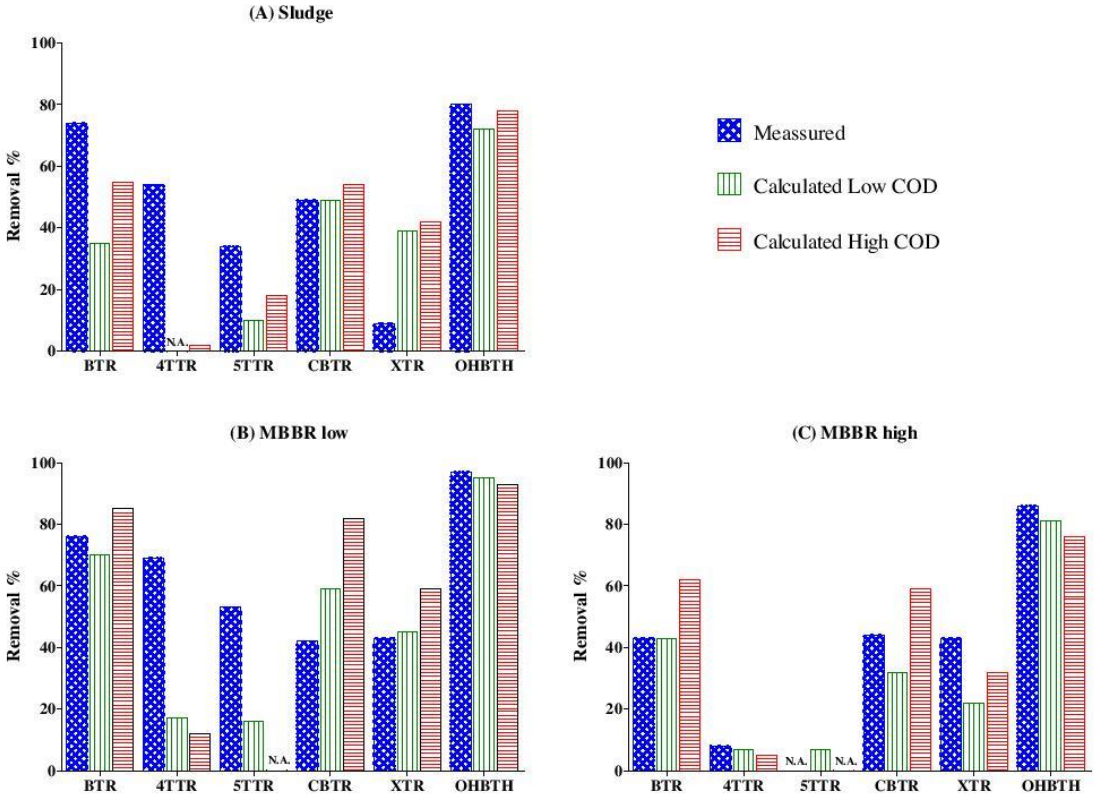

Fig. 4. Measured and calculated removal in AS (A), MBBR-low (B) and MBBR-high system (C).

originating from the high loaded MBBR (MBBR-high). CBTR, XTR and OHBTH had no differences when comparing the potential of BC1 and BC2, while biomass in the second bioreactor had statistically significant greater ability to remove BTR (in MBBR-high, the specific removal rate for 4TTR and 5TTR could not be calculated in BC1). These results indicate that biomass with different ability to remove micropollutants can be developed in each bioreactor of a MBBR system and BC2 seem to have a significant role in the development of microorganisms with higher capability to biodegrade micropollutants. It is known that the development of attached biomass is strongly affected by the wastewater characteristics (pH, temperature, type of bioavailable organic compounds, abundance of nutrients) and the operational conditions of the system (organic loading, aeration rate). The existence of low concentrations of micropollutants could also affect bacterial behavior. In a recent study, it was reported that even small concentration of a xenobiotic compound (0.1 $\mu g\,L^{-1}$ for PFOA and PFOS and 0.5 $\mu g\,L^{-1}$ for triclosan) can provoke increase of extracellular polymers (EPS) in sludge, therefore affecting the transfer of substances from the mixed liquor to the interior of the flocs or the biofilm (Pasquini et al., 2013). This could decrease the amount of micropollutants available to microorganisms and therefore decrease their removal.

3.4. Effect of substrate on biodegradation kinetics

To investigate the role of substrate on biodegradation of target compounds, batch experiments were conducted and biodegradation constants were calculated under high and low COD concentrations (Fig. 3 and Table S3).

For the AS experiment, the biomass used was not taken from the continuous flow system but from the local municipal STP operated at the same SRT (18 d) in order to be able to perform the batch experiment at the same time as the experiment with biomass from the high load MBBR. The biodegradation constants (k_{bio}) calculated for BTR, CBTR, XTR and OHBTH were in the same range as the values found for AS in our previous work (Mazioti et al., 2015). The increased substrate concentration resulted in increased biodegradation (Fig. 3A), indicating that biodegradation of the target compounds in AS is due to co-metabolism by microorganisms utilizing a wide range of carbon sources. Similar observations for the role of co-metabolism have already been reported in previous studies (Huntscha et al., 2014; Mazioti et al., 2015). Biodegradation constants for 4TTR and 5TTR were generally very low during the experiment (24 h). This is in agreement with our previous work (Mazioti et al., 2015) but contradict the ability of the continuous-flow AS system used in this study to degrade these compounds. In the literature, contradictive results have been reported for the biodegradation potential of these two compounds in AS systems. Weiss et al. (2006) and Herzog et al. (2014a) reported no removal of 4TTR during AS process, while Liu et al. (2011) reported that biodegradation of 5TTR was very slow (complete removal after 91 d). Huntscha et al. (2014) reported half-lives of 8.5 d and 0.9 d for 4TTR and 5TTR, respectively. Having in mind that factors such as SRT and increase of substrate concentration do not seem to explain the differences observed in biodegradation of

these two compounds, there is need for further research on the characteristics of biomass and the specific groups of microorganisms involved in their removal.

Concerning the attached biomass, to the best of our knowledge, no biodegradation constants have been calculated for the target compounds so far. First order degradation constants (k) were in the same range with constants calculated for AS (Table S3 and Fig. S4). When normalized to the concentration of biomass, the biodegradation constants (k_{bio}) for the attached biomass were higher, especially for biomass originated from BC2 whereas the concentration of solids was lower (Table S3). Among target compounds, the highest k_{bio} were obtained for CBTR, BTR and OHBTH and were 6.7, 5.6 and 4.8 L g_{SS}^{-1} d^{-1}, respectively. Regarding the role of COD on biodegradation kinetics, similarly to AS experiments, the increase of COD enhanced biodegradation of target compounds (Fig. 3B and C). These results indicate that co-metabolic phenomena are also responsible for the biodegradation of target compounds in attached biomass systems.

3.5. Comparing calculated and predicted removal efficiencies

To investigate how well batch biodegradation kinetics predict the removal of target compounds in continuous-flow systems, Eq. (4) was used to predict the removal of each target compound and the predicted removal efficiencies are compared with measured removal efficiencies as shown in Fig. 4. The predicted removal by AS was very close to the observed removal for CBTR and OHBTH. For XTR, the measured removal was much lower than the predicted, while on the other hand BTR was actually removed at a higher extent (74%) than predicted (35% and 55%). Little removal was predicted for 4TTR and 5TTR which is quite different from that is observed in the continuous-flow system (Fig. 4A). The differences might be due to the fact that the biomass used in batch experiments for the calculation of kinetics was not the same as that used in the continuous flow experiment. These observations indicate that for 4 out of 6 target compounds, care should be given on batch biodegradation kinetics used for predicting their removal in full-scale systems, as the origin of biomass seem to affect the results.

Among MBBR systems, as it was expected, better prediction was achieved for MBBR-high as the biomass used for batch and continuous-flow experiments was the same. Specifically, the behavior of BTR, 4TTR, 5TTR and OHBTH was predicted sufficiently, while minor fluctuations were observed for CBTR and XTR (Fig. 4C). Regarding MBBR-low system, the use of Eq. (4) predicted sufficiently 3 out of 6 (BTR, XTR, OHBTH). However, significant differences were observed especially for 4TTR and 5TTR (Fig. 4B).

4. Conclusions

Both AS and MBBR system were able to biodegrade the target compounds. Removal efficiencies ranged from 43% to 76% for BTR, 8% to 69% for 4TTR, 0% to 53% for 5TTR, 42% to 49% for CBTR, 9% to 43% for XTR and 80% to 97% for OHBTH. The biomass developed in the MBBR system had greater capacity for removal, especially when operated under low organic loading. The presence of easily degradable organic matter enhanced biodegradation of compounds in batch tests. Further research is needed especially for 4TTR and 5TTR, focusing on specific microorganisms that could be responsible for their biodegradation.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.06.035.

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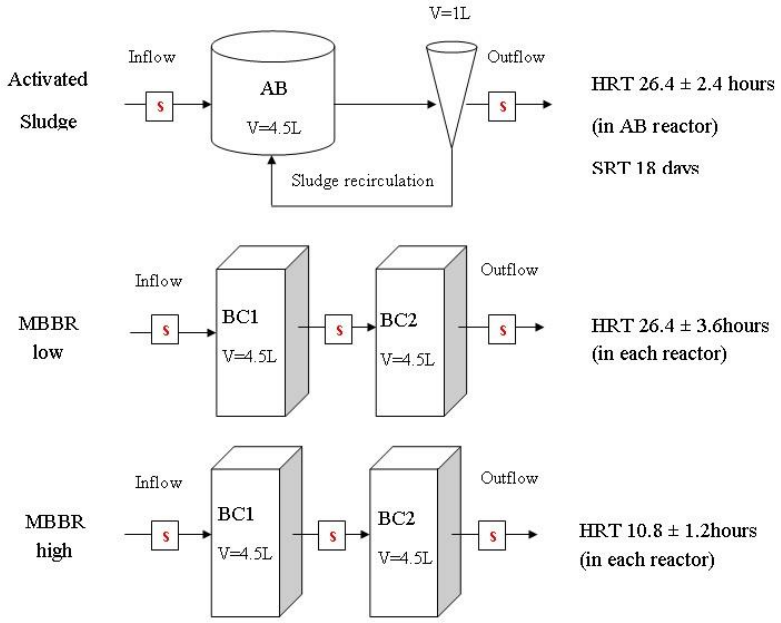


Figure S1. Schematic description of the continuous-flow biological treatment systems used in this study (sampling points are presented with an S).

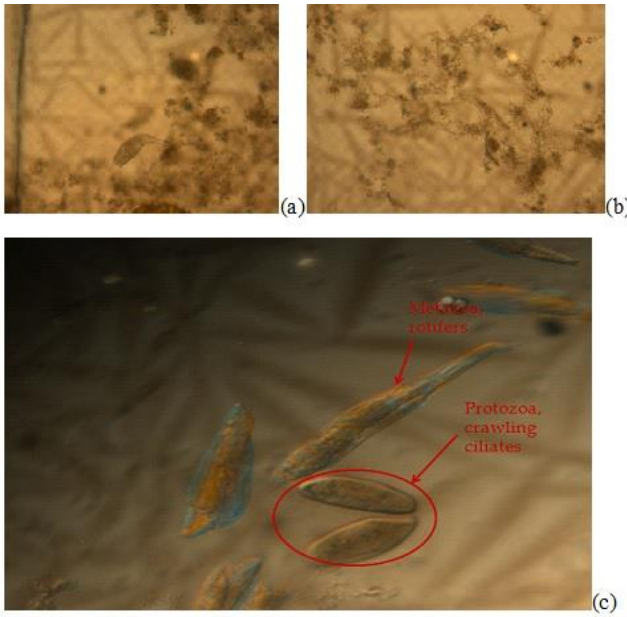


Figure S2. Sample images from the microscopic observations conducted in AS system (a. rotifer, b. filamentous bacteria) and MBBR system (c. rotifers and protozoa).

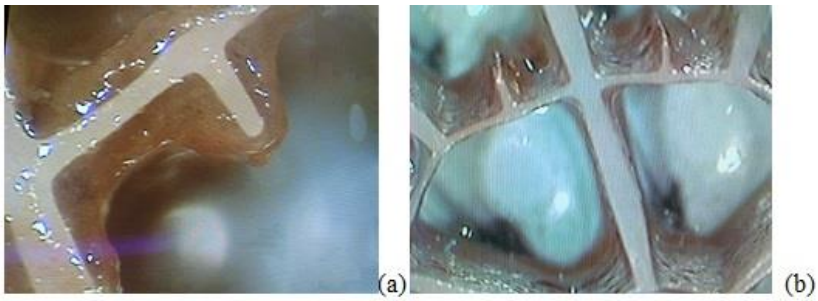


Figure S3. Biofilm formation on carriers in BC1 (a) and BC2 (b) in the MBBR-low system.

Table S1. Characteristics of raw wastewater used in the current study (n = 30, standard deviations are given in parentheses).

Parameter	Value
pH	7.3 (±0.3)
COD_{dis} (mg L^{-1})	272 (±179)
NH_4-N (mg L^{-1})	50 (±16)
NO_3-N (mg L^{-1})	3.2 (±2.5)
TSS (mg L^{-1})	86 (±44)

Table S2. Mass of micropollutants removed per mass of biomass and day during continuous flow experiments with Biocarriers under low loading conditions (MBBR-low) and Biocarriers under high loading conditions (MBBR-high) in each bioreactor (BC1 and BC2). Removal of 4TTR and 5TTR in MBBR-low (BC1) was very low and the relevant values were not calculated (N.A.).

System/Reactor		Specific removal (μg of micropollutant removed per g of biomass per day)					
		BTR	4TTR	5TTR	CBTR	XTR	OHBTH
MBBR-low	BC1	11.9 (±1.3)	9.7 (±3.6)	10.3(±2.8)	3.9 (±1.7)	3.5(±1.6)	13.6 (±2.2)
	BC2	8.8 (±4.6)	11.9(±8.5)	14.4(±9.9)	11.3(±6.5)	9.7(±3.3)	3.1(±2.6)
MBBR-high	BC1	4.4 (±2.0)	N.A.	N.A.	6.5(±2.1)	4.1(±2.9)	12.2 (±3.2)
	BC2	11.0 (±5.3)	15.1 (±12.3)	6.1 (±5.3)	6.4(±7.1)	5.5(±6.4)	8.9 (±5.6)

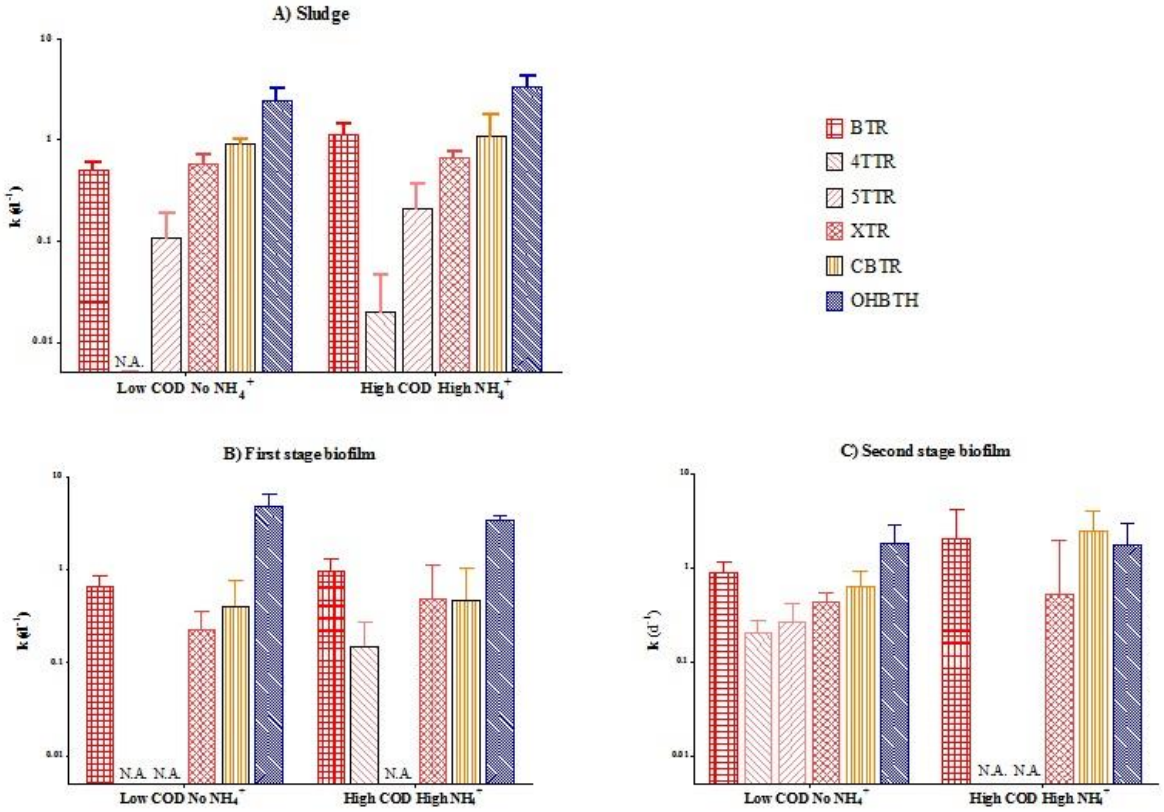


Figure S4. Effect of low or high COD concentrations on observed biodegradation constants, k (d^{-1}) in batch experiments with activated sludge (A) and attached biomass from BC1 (B) and BC2 (C) (t-bars represent 95% confidence interval).

Table S3. Biodegradation constants calculated during batch experiments with suspended and attached biomass, under low and high COD concentrations (average values and standard deviation).

Biodegradation rate constant, k (d^{-1})													
Experiment	COD	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.
		BTR		**4TTR**		**5TTR**		**CBTR**		**XTR**		**OHBTH**	
BC1[1]	Low[4]	**0.66**	0.21	**N.A.**	N.A.	**N.A.**	N.A.	**0.41**	0.37	**0.22**	0.14	**4.74**	1.62
BC1[1]	High[5]	**0.98**	0.33	**0.15**	0.12	**N.A.**	N.A.	**0.48**	0.56	**0.49**	0.61	**3.43**	0.44
BC2[2]	Low[4]	**0.90**	0.26	**0.20**	0.08	**0.27**	0.16	**0.64**	0.30	**0.43**	0.12	**1.82**	1.06
BC2[2]	High[5]	**2.03**	2.22	**N.A.**	N.A.	**N.A.**	N.A.	**2.43**	1.64	**0.53**	1.46	**1.78**	1.17
AB[3]	Low[4]	**0.50**	0.11	**N.A.**	N.A.	**0.11**	0.09	**0.90**	0.13	**0.58**	0.12	**2.41**	0.78
AB[3]	High[5]	**1.11**	0.32	**0.02**	0.01	**0.21**	0.16	**1.07**	0.74	**0.68**	0.08	**3.36**	0.94
Pseudo first-order biodegradation rate constant, k_{bio} (L g_{SS}^{-1} d^{-1})													
Experiment	COD	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.	average	st.dev.
		BTR		**4TTR**		**5TTR**		**CBTR**		**XTR**		**OHBTH**	
BC1[1]	Low[4]	**0.58**	0.18	**N.A.**	N.A.	**N.A.**	N.A.	**0.36**	0.33	**0.17**	0.10	**3.84**	1.31
BC1[1]	High[5]	**0.86**	0.29	**0.12**	0.10	**N.A.**	N.A.	**0.43**	0.50	**0.37**	0.46	**2.78**	0.35
BC2[2]	Low[4]	**2.46**	0.71	**0.54**	0.20	**0.72**	0.42	**1.77**	0.82	**1.11**	0.31	**4.86**	2.84
BC2[2]	High[5]	**5.58**	6.08	**N.A.**	N.A.	**N.A.**	N.A.	**6.72**	4.54	**1.39**	3.81	**4.77**	3.14
AB[3]	Low[4]	**0.31**	0.06	**N.A.**	N.A.	**0.06**	0.05	**0.57**	0.08	**0.28**	0.06	**1.30**	0.42
AB[3]	High[5]	**0.68**	0.19	**0.01**	0.01	**0.12**	0.09	**0.67**	0.47	**0.32**	0.04	**1.82**	0.51

[1]BC1: Bioreactor with Biocarriers 1 collected from MBBR high; [2]BC2: Bioreactor with Biocarriers2 collected from MBBR high;[3]AB: Aerobic Bioreactor with activated sludge collected from Mytilene's STP; [4]Low COD: initial concentration: 28 (±15) mg L^{-1};[5]High COD: initial concentration: 272 (±107) mg L^{-1}

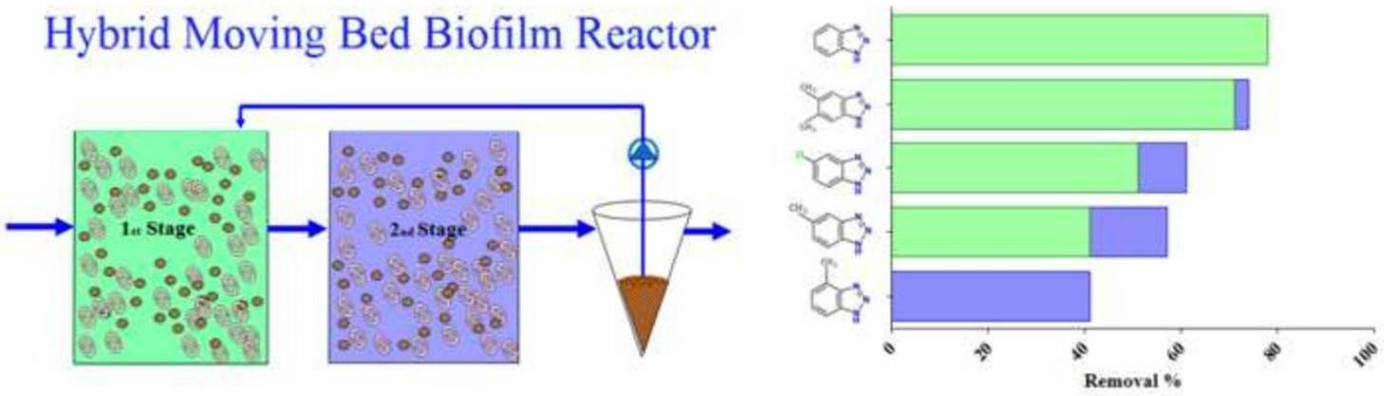
Paper C (submitted for publication)

*Highlights (for review)

Highlights

- All target compounds were partially removed in hybrid moving bed biofilm reactor
- 5 compounds were removed mainly in 1st stage, critical role of 2nd stage for 4TTR
- AS and biocarriers contribute to different extent to micropollutants biodegradation
- HMBBR and low loaded MBBR are the most efficient systems for studied compounds
- 22 biotransformation products were tentatively identified

Graphical Abstract



Hybrid Moving Bed Biofilm Reactor for the biodegradation of benzotriazoles and hydroxy-benzothiazole in wastewater

Aikaterini A. Mazioti[a], Athanasios S. Stasinakis[a,b*], Aikaterini K. Psoma[c], Nikolaos S. Thomaidis[c], Henrik R. Andersen[b]

[a]Department of Environment, University of the Aegean, 81100 Mytilene, Greece

[b]Department of Environmental Engineering, Technical University of Denmark, Miljøvej, B 113, 2800 Kgs. Lyngby, Denmark

[c]Department of Chemistry, National and Kapodistrian University of Athens, Panepistimioupolis Zografou, 15771 Athens, Greece

*Corresponding author. Tel.: +30 22510 36257; fax: +30 22510 36206

E-mail address: astas@env.aegean.gr

18 **ABSTRACT**

19 A laboratory scale Hybrid Moving Bed Biofilm Reactor (HMBBR) was used to study the
20 removal of five benzotriazoles and one benzothiazole from municipal wastewater. The HMBBR
21 system consisted of two serially connected fully aerated bioreactors that contained activated
22 sludge (AS) and K3-biocarriers and a settling tank. The average removal of target compounds
23 ranged between 40% (4TTR) and 80% (OHBTH) and, except for 4TTR, degradation mainly
24 occurred in the first bioreactor. Calculation of biodegradation constants in batch experiments and
25 application of a model for describing micropollutants removal in the examined system showed
26 that AS is mainly involved in biodegradation of OHBTH, BTR and XTR, carriers contribute
27 significantly on 4TTR biodegradation, while both types of biomass participate on elimination of
28 CBTR and 5TTR. Comparison of the HMBBR system with MBBR or AS systems from
29 literature, showed that for the same operational conditions (organic loading, hydraulic retention
30 time), the HMBBR system was more efficient for the biodegradation of the investigated
31 chemicals. Biotransformation products of target compounds were identified using ultra high-
32 performance liquid chromatography, coupled with a quadrupole-time-of-flight high-resolution
33 mass spectrometer (UHPLC-QToF-MS). The samples were screened for potential transformation
34 products according to a suspect database and twenty two biotransformation products were
35 tentatively identified, while retention time denoted the formation of more polar transformation
36 products than the parent compounds.

37

38 **Keywords:** emerging pollutants; biological treatment; sewage; kinetics; biotransformation;
39 biofilm

1. Introduction

Growing demand for more efficient wastewater treatment is leading to new technologies for treatment as well as improvement of existing ones. Concerning biological treatment, the Hybrid Moving Bed Biofilm Reactor (HMBBR) is an approach that was introduced two decades ago for the first time in wastewater engineering (Randall and Sen, 1996). The HMBBR is a combination of a typical activated sludge (AS) system with a Moving Bed Biofilm Reactor (MBBR), in which biofilm attached on biocarriers and AS flocs co-exist in the bioreactor, contributing to wastewater treatment. The main advantages of such a system compared to AS are the lower requirement for process volume, the increased nitrification capacity and the lower sludge load on the secondary clarifier (Di Trapani et al., 2013). Due to the above, HMBBR systems have been successfully used for upgrading of conventional AS systems (Mannina and Viviani, 2009; Di Trapani et al., 2011).

Beside the above, so far, only few studies have focused on the ability of HMBBR systems to remove micropollutants from wastewater. Falås et al. (2013) examined the elimination of 20 micropollutants from a large scale HMBBR in Switzerland and reported that the attached growth biomass can contribute significantly to the removal of specific compounds in such systems. Escolà Casas et al. (2015) investigated the removal of 26 pharmaceuticals in hospital wastewater by a 4 staged pilot treatment plant consisting of AS, HMBBR and MBBR reactors in series and reported biodegradation kinetics in different bioreactors. Finally, Sfaelou et al. (2015) recently examined the effects and removal of phenanthrene in sequencing batch reactors containing AS and biocarriers. To the best of our knowledge, no other studies have been published on the removal of micropollutants in HMBBR systems.

62 Benzotriazoles (BTRs) and Benzothiazoles (BTHs) are two groups of micropollutants that
63 occur in wastewater from domestic and industrial activities (Farré et al, 2008). BTRs are found
64 in corrosion-inhibiting products, cooling fluids, de-icing fluids and dishwashing detergents
65 (Reemtsma et al., 2010), while BTHs are used as vulcanization accelerators and stabilizers in the
66 photo industry (Herrero et al., 2014). Both groups are highly soluble in water and highly polar,
67 leading to their persistence in the water cycle (Reemtsma et al., 2006; Nödler et al., 2014). The
68 partial removal of some of them in AS systems has been documented in monitoring studies
69 (Asimakopoulos et al., 2013; Stasinakis et al., 2013; Molins-Delgado et al., 2015) and laboratory
70 biodegradation experiments (Liu et al., 2011; Mazioti et al., 2015a). Moreover, information on
71 the biotransformation products of specific BTRs (1H-benzotriazole, BTR; 4-methyl-1H-
72 benzotriazole, 4TTR; 5-methyl-1H-benzotriazole, 5TTR) has been reported in activated sludge
73 experiments (Liu et al., 2011; Huntscha et al., 2014). In a recent study, Mazioti et al. (2015b)
74 compared the ability of AS and pure MBBR systems to biodegrade six of these compounds
75 (BTR; 4TTR; 5TTR; xylytriazole, XTR; 5-chlorobenzotriazole, CBTR; 2-hydroxybenzothiazole,
76 OHBTH) and reported that attached biomass had higher biodegradation potential compared to
77 AS. To the best of our knowledge, no information is available on the removal of these
78 compounds in HMBBR and on the contribution of co-existing types of biomass on their
79 biodegradation.
80 The aim of this study was to investigate the potential of a laboratory scale HMBBR system,
81 consisting of two bioreactors in series, to remove BTR, 4TTR, 5TTR, XTR, CBTR and OHBTH
82 from domestic wastewater. Concentrations of target compounds in different points of the hybrid
83 system were monitored and the observed removal efficiencies were compared with those
84 reported in a previous study using AS and MBBR systems (Mazioti et al., 2015b).

85 Biodegradation kinetics of the target compounds were also determined using AS and biocarriers
86 from the HMBBR system and a model was applied to describe the contribution to
87 micropollutants removal by different mechanisms (biodegradation, sorption) and by different
88 types of biomass (sludge, biofilm). Finally, batch experiments were conducted and for the first
89 time biotransformation products formed in a HMBBR reactor were tentatively identified.
90

91 **2. Materials and Methods**

92 **2.1. Analytical standards and reagents**

93 Analytical standards of XTR and CBTR were supplied by Sigma-Aldrich (USA). BTR was
94 purchased from Merck (Germany), 4TTR by Fluka (Switzerland), 5TTR by Acros Organics
95 (Belgium); whereas OHBTH was purchased from Alfa Aesar (USA). Stock solutions of
96 individual compounds were prepared in methanol (MeOH) at 1000 mg L^{-1} and kept at −18 °C.
97 Working solutions of 10 mg L^{-1} were prepared when needed and were kept at −18 °C for a time
98 period not exceeding three months. Methanol (MeOH, HPLC-MS grade) and acetonitrile (ACN,
99 HPLC grade) were purchased from Merck (Germany) and Fisher (USA), respectively. The solid
100 phase extraction (SPE) cartridges used for samples' clean-up were polymer-based with surface
101 modified styrene divinylbenzene phase (Strata-X, 33u Polymeric Reversed Phase 200mg/6ml)
102 and they were supplied by Phenomenex (USA). HPLC grade water was prepared in the
103 laboratory using a MilliQ/MilliRO Millipore system (USA). Ultra-pure HCl (32%), used for
104 samples acidification, was purchased from Merck (Germany).
105

106 **2.2. Continuous flow systems: set-up and operation**

107 A small scale continuous flow system was installed and operated in the laboratory (Figure 1),
108 under constant room temperature controlled by central air-conditioning system. The HMBBR
109 system consisted of two aerobic bioreactors (BC1 and BC2) connected in series, with a working
110 volume of 3 L each. A settling tank, with a volume of 1 L, followed the BC2, from which AS
111 was recirculated to BC1. Each bioreactor contained both biocarriers (type K3, AnoxKaldnes, at a
112 filling ratio of 30%) and AS. The AS was collected from a nitrifying municipal STP (Mytilene,
113 Greece), while the biocarriers were taken from a laboratory scale MBBR system that has been
114 operated for several months and on which a mature biofilm was attached (Mazioti et al., 2015b).
115 A hydraulic residence time (HRT) of 12.4 ± 0.6 h (for each reactor) was applied, providing a
116 substrate organic loading equal to 0.64 ± 0.39 kg m^{-3} d^{-1} for BC1 and 0.11 ± 0.09 kg m^{-3} d^{-1} for
117 BC2; whereas sludge residence time (SRT) of AS in the system was kept at 8 d. The HMBBR
118 system was fed with raw wastewater collected from the STP of the University Campus in
119 Mytilene, Greece (Table S1). In all bioreactors, the conservation of aerobic conditions and the
120 adequate mixing of suspended and attached biomass were achieved by providing constant air
121 supply, which ensured that the dissolved oxygen concentration (DO) was always higher than 4
122 mg L^{-1}.

123 An acclimatization period of 27 days took place (time almost equal to three times SRT),
124 during which conventional pollutants removal (Chemical Oxygen Demand, COD; NH_4-N),
125 concentration of suspended and attached biomass and values of pH, temperature (T) and DO
126 were frequently examined in order to control the system's stability and efficiency. Afterwards,
127 the target compounds were spiked to the raw wastewater using methanol solutions to obtain a
128 daily stable inflow concentration of approximately 20 μg L^{-1} of each investigated chemical. To

129 evaluate the removal of the target compounds in different bioreactors, samples were taken during
130 one week from different sampling points of the system (Figure 1).
131
132 **2.3. Batch biodegradation experiments for kinetics calculation**
133 To determine the contribution of each type of biomass in the removal of target compounds,
134 batch experiments were conducted and biodegradation kinetics was calculated. For this reason,
135 four days after the end of spiking micropollutants to the HMBBR system (time equal to almost
136 eight HRTs), AS and biocarriers were taken from BC1 and BC2 and separate batch experiments
137 were conducted for each of the two types of biomass. All experiments were conducted in
138 stoppered glass bottles that were constantly shaken. The working volume in each reactor was 1 L
139 and aeration was constantly provided through porous ceramic diffusers. The initial wastewater
140 parameters in each flask were similar to those normally found in the bioreactors (Table S2). The
141 investigated compounds were spiked in methanol solutions to obtain an initial concentration of
142 approximately 30 μg L^{-1} for each investigated chemical in the reactors. To quantify the
143 biodegradation of the target chemicals, homogenized samples (50 mL) were collected after 0, 1,
144 2.5, 5, 12 and 24 hours. Since sorption to organic matter is of minor importance for these
145 compounds (Mazioti et al., 2015a), the concentrations of target compounds were determined
146 only in the dissolved phase using the analytical method described in Paragraph 2.5.
147
148 **2.4. Batch biodegradation experiments for biotransformation products identification**
149 To identify the biotransformation products of target compounds in the HMBBR system,
150 aerated batch experiments were conducted using biomass from BC1 where the greatest part of
151 biodegradation was observed during the continuous flow experiment. Mixture of AS and

152 biocarriers from BC1 was transferred to seven different glass bottles at a final volume of 200
153 mL. Each target compound was spiked in a different bottle at an initial concentration of 10 mg L^{-1},
154 while a control flask was also prepared containing biomass and methanol at an amount equal to
155 that added in other reactors. All bottles were covered with aluminium foil and constantly agitated
156 on a shaking plate. The total duration of the experiment was 24 h. Three homogenized samples
157 (10 mL each) were taken from each reactor at 0, 6 and 24 h.
158
159 **2.5 Analytical methods**
160 Analysis of COD, NH_4-N, NO_3-N, Total Suspended Solids (TSS) and Mixed Liquor
161 Suspended Solids (MLSS) were performed according to Standard Methods (APHA, 1998),
162 temperature, DO and pH were measured using portable instruments. The quantification of the
163 attached biomass was performed by removing the biofilm from biocarriers and measuring the
164 dried weight difference, as described by Falås et al. (2012).
165 For the investigation of target compounds fate, samples were filtered through glass fibre
166 filters (GF-3 Macherey Nagel). Filtrates were collected, acidified to pH 3.0 ± 0.1 and stored at 4
167 ^{o}C until analysis. Analysis of target compounds in the dissolved phase was based on previously
168 developed methods (Asimakopoulos et al., 2013; Mazioti et al. 2015) and included solid phase
169 extraction (SPE). Chromatographic analysis was performed by a Shimatzu (Japan) LC20-AD
170 prominence liquid chromatographer associated with a SPD-M20A prominence diode array
171 detector and a SIL-20AC auto sampler. Satisfactory recoveries and precision of the analytical
172 procedure was achieved; where the obtained LODs ranged from 17 ng L^{-1} (BTR) to 125 ng L^{-1}
173 (CBTR). Further information for the analytical method and the chromatographic conditions can
174 be found in a recently published paper (Mazioti et al., 2015a).

For the investigation of transformation products, samples were initially filtered through glass fibre filters (GF-3 Macherey Nagel), 1.5 mL of each sample was filtered through 0.2 µm RC filter and collected. Filtrates were stored at -18^{o}C until analysis. A LC-HR-MS/MS analysis Ultrahigh-performance liquid chromatography (UHPLC) system (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Germany), coupled with a quadrupole-time-of-flight high-resolution mass spectrometer (UHPLC-QToF-MS) (Maxis Impact QTOF, Bruker, Bremen, Germany) was used for transformation products identification. The chromatographic separation was performed using a Thermo Acclaim RSLC C18, 2.2 µm 120 Å, 2.1 x 100 mm column. The gradient program for both positive and negative mode is presented in Table S3. Methanol (solvent A) and water:methanol (90:10) (solvent B) both amended with 0.01% formic acid and 5 mM ammonium formate was used as mobile phase for positive ionization and methanol and water:methanol (90:10) both amended with 5 mM ammonium acetate as an eluent for negative ionization mode. A sodium formate solution (10 mM) was always introduced between 0.1 to 0.3 min in the beginning of every chromatographic run through direct infusion at a flow rate of 50 µL h^{-1} to compensate for mass drifts and for internal mass calibration. Sodium formate solution was also used to perform daily external calibration in QTOFMS. The sodium formate calibration mixture consists of 10 mM sodium formate in a mixture of water/isopropanol (1:1).

The QToF mass spectrometer was equipped with an electrospray ionization interface (ESI) operating both in positive and negative ionization mode. Operation parameters were: capillary voltage, 2500 V; end plateoffset, 500 V; nebulizer pressure, 2 bar (N_2); drying gas, 8 L min^{-1} (N_2); and drying temperature, 200 °C. Data were acquired through broad-band collision induced dissociation (bbCID) mode, providing MS and MS/MS spectra simultaneously under positive and negative electrospray ionization (two separate runs). HR-MS data was recorded within a

198 mass-to-charge (m/z) range of 50–1000 for each sample, at 2 Hz spectra rate and at a
199 continuously alternatively collision energy of 4 eV (low energy, LE) and 25 eV (high energy,
200 HE) in the collision cell Q2, for full-scan and MS/MS data, respectively. For masses
201 corresponding to plausible transformation products (TPs), the fragmentation performed in Auto
202 MS/MS mode with an inclusion list. For masses corresponding to the detected plausible
203 transformation products (TPs), MS/MS spectra was subsequently acquired with data dependent
204 acquisition in Auto MS/MS mode with an inclusion list.

205 For TPs' identification, the samples were screened for the exact masses of potential TPs
206 according to a suspect database that was compiled by the online pathway prediction system
207 hosted by EAWAG institute (EAWAG-PPS) without the "relative reasoning mode". Two
208 generations of TPs for each BTR and OH-BTH were predicted. MetabolitePredict (Bruker,
209 Bremen, Germany), was also used for the prediction of possible phase I & II metabolites as well
210 as cytochrome P450 metabolites, to extend the possible candidates for screening (Bletsou et al.,
211 2015). For instance, monohydroxylation of benzotriazoles is not predicted by EAWAG-PPS, but
212 it is predicted by MetabolitePredict software. Finally, already known and reported metabolites
213 from the literature were added to the database (Liu et al., 2011; Huntscha et al., 2014).

214 A data-processing software (TargetAnalysis 1.3, Bruker) was used for the suspect screening
215 of plausible transformation products. All the time interval samples were screened, in both
216 positive and negative ionization modes, for the determination of suspect TPs from the database.
217 The characterization of an exact mass as a possible TP was based on the following criteria,
218 deltaRT $\leq$ 0.10 min, mass error $\leq$ 5 ppm, isotopic fit: $\leq$ 1000 mSigma, intensity threshold >500
219 (+ESI) and >200 (-ESI) as well as, absence from the blank samples and occurrence of a time
220 trend (Li et al., 2013). The potential TPs were subjected to MS/MS experiments via AutoMS

mode with an inclusion list in order to obtain the MS/MS spectra and the fragments for further assignment of molecular formulas and structure elucidation. The SmartFormula algorithm was used to apply the sum formulae of the protonated or deprotonated ion and fragments (mass error and isotopic fit was also calculated). SmartFormula uses element restrictions for C, H, N and O, $[M\pm H]^{\pm}$ for positive and negative ion mode, mass tolerance of 5 ppm, the hydrogen to carbon ratio (H/C) ranges from 0 to 3, it checks for ring and double bonds and allows even electron configuration for the MS peaks and both odd and even electron configuration for MS/MS peaks.

2.6 Equations

Micropollutants removal in laboratory scale reactors was calculated according to Eq. (1):

$$Removal = \left(1 - \frac{C_{out}}{C_{in}}\right) \times 100 \quad (1)$$

Where C_{in} is the concentration of target compound in influent wastewater (μg L^{-1}) and C_{out} is the concentration in treated wastewater for each examined reactor (μg L^{-1}).

Specific removal rate (as μg of micropollutant removed per g of biomass per day) for each compound was calculated according to Eq.(2):

$$Specific\ Removal\ Rate = \left(\frac{C_{in}Q_{in} - C_{out}Q_{out}}{X \times V}\right) \quad (2)$$

Where Q_{in} and Q_{out} are the flow rates of influent and effluent wastewater, respectively (L d^{-1}), X is the total (attached + suspended) concentration of biomass (g L^{-1}) and V is the volume of each bioreactor (L).

The biodegradation rate constants (k) were estimated using first order kinetics. Pseudo first-order biodegradation rate coefficient, k_{bio}, normalized to attached or suspended biomass (L g^{-1} d^{-1}) was calculated for each biodegradation experiment using the appropriate sorption constant (K_d; L g^{-1}) for each compound (Mazioti et al. 2015a) and Eq. (3) (Ziels et al. 2014):

$$ln\frac{C_t}{C_0} = -k_{bio} \times \left(\frac{X}{1+K_dX}\right) \times t \qquad (3)$$

Where C_t and C_0 are the dissolved target compound concentrations in batch experiment at time t and t = 0, respectively (μg L^{-1}).

In order to predict the removal of target compounds in each bioreactor and determine the role of each type of biomass on their elimination, Equations 4 and 5 were used (Tchobanoglous et al., 2002) for the existing HMBBR system:

$$M_{in} = M_{BC1-car.} + M_{BC1-sl.} + M_{BC2-car.} + M_{BC2-sl.} + M_{sorbed} + M_{out} \qquad (4)$$

Where M_{in} and M_{out} are the masses of target compounds in influent and effluent wastewater, respectively (μg d^{-1}), $M_{BC1-car.}$ and $M_{BC1-sl.}$ are the masses of target compounds that are biodegraded in BC1, by the attached (carriers) and suspended (AS) biomass respectively (μg d^{-1}), $M_{BC2-car.}$ and $M_{BC2-sl.}$ are the masses of target compounds that are biodegraded in BC2, by the attached (carriers) and suspended (AS) biomass respectively (μg d^{-1}) and M_{sorbed} is the mass of each target compound removed with excess sludge from the bioreactors (μg d^{-1}).

$$C_{in}Q_{in} = (k_{bio-car.}C_{out}X_{car.}V + k_{bio-sl.}C_{out}X_{sl.}V)_{BC1}$$
$$+(k_{bio-car.}C_{out}X_{car.}V + k_{bio-sl.}C_{out}X_{sl.}V)_{BC2} + \frac{(X_{sl.}VK_dC_{out})}{SRT} + (Q_{out}C_{out}) \qquad (5)$$

Where C_{in} and C_{out} are the concentrations of target compounds in influent and effluent wastewater, respectively (μg m^{-3}), Q_{in} and Q_{out} are the flow rates in influent and effluent wastewater, respectively (m^3 d^{-1}), $k_{bio-car.}$ and $k_{bio-sl.}$ are the normalized biodegradation constants for attached and suspended biomass, respectively (L g^{-1} d^{-1}), as calculated in batch experiments for the loading conditions existing in the two reactors (BC1 and BC2), $X_{car.}$ and $X_{sl.}$ is the concentration of attached biomass on carriers and the concentration of MLSS, respectively (g L^{-1}). Furthermore, V is the volume of each reactor (m^3), K_d is the sludge-water

distribution coefficient (L g^{-1}), as calculated in a previous work (Mazioti et al., 2015a) and SRT is the sludge residence time in the system (d).

2.7 Statistical analysis

In order to compare the removal values and specific removal rates one-way ANOVA was used with the Tukey-Kramerpost-test in order to determine significant differences between groups.

3. Results and Discussion

3.1. Operation of continuous flow HMBBR system

The HMBBR system was stable during the whole experimental period (34 d) and achieved sufficient removal of dissolved COD (87%) and NH_4-N (98%) (Figure 1). The major part of conventional pollutants was removed in BC1, whereas the use of BC2 improved further the quality of treated wastewater decreasing the average concentrations of COD_{dis} and NH_4-N to 24 mg L^{-1} and 1 mg L^{-1}, respectively. As it was expected due to sludge recirculation, the concentrations of activated sludge were almost the same in both bioreactors. On the other hand the increased organic loading into BC1 resulted in a higher concentration of attached biomass (1023 ± 165 mg L^{-1}) comparing to that observed in BC2 (610 ± 198 mg L^{-1}).

3.2. Removal of target compounds in continuous flow HMBBR system

The HMBBR system exhibited significant decreases of all the target compound concentrations in wastewater (Figure 2), resulting in average removals ranging between 40% (4TTR) and 80% (OHBTH). The observed decrease of micropollutants concentration was mainly

287 due to biodegradation as it is known that these compounds are not degraded abiotically in STPs
288 and they are poorly sorbed to biomass (Mazioti et al. 2015a). Except for 4TTR, all investigated
289 chemicals were removed in BC1, while the second bioreactor (BC2) did not statistically
290 significantly improve their removal. The removal of most target compounds in BC1 where there
291 was a higher COD concentration indicates the role of co-metabolism in the compounds
292 biodegradation. Similar observations for the co-metabolic degradation of these target compounds
293 were also described in previous studies (Mazioti et al., 2015a, b). Concerning 4TTR, it seems
294 that the biomass grown in BC2 had the ability to biodegrade it, whereas this property was not
295 present in BC1. So far, in the literature contradictory results have been reported for
296 biodegradation of 4TTR and 5TTR in AS and MBBR systems, indicating the important role of
297 biomass used and the role of specific microorganisms on its removal (Weiss et al., 2006; Herzog
298 et al., 2014; Mazioti et al., 2015b).

299 Comparison of the removal efficiency of target compounds in the HMBBR system with those
300 previously observed in pure MBBR and AS systems (Mazioti et al., 2015a, 2015b) showed that
301 the current system achieved similar or statistically higher elimination for 4 out of 6 examined
302 chemicals (Figure 3a). Only OHBTH and 4TTR were removed more efficiently in a pure MBBR
303 system that operated under lower organic loading conditions (0.25 kg m^{-3} d^{-1} in the first stage
304 and 0.05 kg m^{-3} d^{-1} in the second stage) and double HRT. It is worth mentioned that when the
305 performance of the HMBBR system is compared with that of a pure MBBR system operated
306 under similar organic loading and HRT conditions (MBBR-high, Figure 3a), a statistically
307 significant increase of removal is observed for 5 out of 6 target compounds, indicating the
308 advantage of the hybrid system on micropollutants removal comparing to a pure MBBR system
309 operated under the same conditions. Finally, the hybrid system achieved statistically higher

310 removal efficiencies for XTR and 5TTR and similar removal for the other compounds comparing
311 to an AS system operated at the double HRT and the same concentration of suspended biomass
312 (Figure 3a). In a previous study, Di Trapani et al. (2010) reported that HMBBR systems can
313 achieve similar performance in terms of organic and nitrogen removal as a traditional AS system
314 operating at lower hydraulic loading, however, to the best of our knowledge, this it is the first
315 time that this is described for micropollutants removal. The efficient performance of a HMBBR
316 system under higher loadings comparing to traditional AS systems could significantly decrease
317 the operational costs of STPs as it is known that the energy consumption for aeration of AS tanks
318 contribute to 40-75% of the total energy requirements in Sewage Treatment Plants (Mamais et
319 al., 2015).

320 In order to clarify if the higher removal of micropollutants in the HMBBR system is due to
321 the biomass properties or to the higher total amount of biomass in such a system, the specific
322 removal expressed as μg of micropollutant per g of biomass per d was calculated for each
323 compound and compared to values reported by Mazioti et al. (2015b) for pure MBBR and AS
324 systems (Figure 3b). No statistical differences (except for XTR) were observed on the ability of
325 HMBBR biomass and AS biomass to remove target compounds. On the other hand, biomass
326 developed in pure MBBR systems showed statistically significant higher specific removal for
327 most target compounds indicating the presence of more efficient bacteria for biodegradation of
328 micropollutants in biofilm developed in a pure MBBR system compared to the HMBBR system.
329 So far, no comparison has been done on the diversity of microorganisms grown on biofilm of
330 hybrid and pure MBBR systems and on their potential to remove micropollutants.

331

332 **3.3 Biodegradation kinetics of attached and suspended biomass of HMBBR system**

333 Batch experiments were conducted to determine the first order rate constant, k, and
334 normalised rate constant, k_{bio}, for each types of biomass (AS, attached biomass on biocarriers)
335 from BC1 and BC2. The highest biodegradation constants were calculated for OHBTH, whereas
336 4TTR and 5TTR exhibited slow degradation (Table S4).
337 Different normalised biodegradation constants were calculated for the two types of biomass
338 contained in the same bioreactor, indicating the significant role of both types of biomass on the
339 removal of this group of micropollutants in a HMBBR system (Figure 4). Specifically in BC1,
340 OHBTH and BTR were biodegraded more rapidly by AS, whereas the opposite was observed for
341 CBTR. Additionally in BC2, higher k_{bio} were calculated for OHBTH, BTR, XTR and CBTR by
342 attached biomass.
343 Comparing the biodegradation kinetics obtained for the same type of biomass in different
344 bioreactors of HMBBR system, in experiments with AS lower k_{bio}'s were calculated for
345 OHBTH, BTR, XTR and CBTR in BC2 (Figure 4, Table S4). As mentioned in paragraph 2.3 and
346 Table S2, batch experiments with biomass from BC2 were conducted under lower organic
347 substrate concentration comparing to those with biomass from BC1 in order to simulate the
348 conditions in the continuous-flow system and be able to afterwards use the calculated constants
349 for model development. Having in mind that the biodegradation of the target compounds by AS
350 is co-metabolic (Mazioti et al., 2015a) and AS recirculates in the system, the lower k_{bio} values
351 observed in BC2 are possibly due to the experimental conditions (lower COD) applied in these
352 batch experiments. Concerning the attached biomass, similar biodegradation constants were
353 calculated for OHBTH, BTR, XTR, CBTR and 5TTR in both bioreactors (Figure 4). As co-
354 metabolic biodegradation of these compounds has also been reported for the attached biomass
355 (Mazioti et al., 2015b), it is likely that the higher COD concentration that was used in the

356 experiments with biomass from BC1 increased to some extent the observed biodegradation rates.
357 Based on the above, it can be assumed that if similar concentrations of COD had been used in
358 both batch experiments, k_{bio} in BC1 would be lower compared to those in BC2.

359 Comparison of the biodegradation constants obtained in this study with k_{bio} values calculated
360 in a previous study (Mazioti et al., 2015b) using attached biomass from a pure MBBR system
361 and AS from a conventional AS system (Figure 4) shows that except for OHBTH among all
362 bioreactors higher biodegradation constants were obtained in the 2nd bioreactor of the pure
363 MBBR system. This observation indicates that in the biofilm of a pure MBBR system there is the
364 potential to develop more specialised microorganisms for biodegradation of micropollutants.
365

366 **3.4. Contribution of different types of biomass to target compounds removal**

367 The removal of target compounds in the HMBBR system was predicted using batch
368 biodegradation kinetics and Equation 5 (Figure 5). Despite the underestimation of removal
369 efficiencies that was observed for some of the target compounds especially in the first reactor
370 (BC1), the applied model described sufficiently the order of removal of studied micropollutants
371 in HMBBR system. Concerning the contribution of different types of biomass to the target
372 compounds removal, it seems that biodegradation by AS occurring in BC1 is the major
373 mechanism for OHBTH, BTR and XTR. Both biocarriers and AS of BC1 and BC2 contribute
374 significantly on biodegradation of CBTR and 5TTR, whereas the attached biomass on biocarriers
375 of BC2 has critical role for 4TTR biodegradation. As it was expected due to the hydrophilicity of
376 these compounds, the role of sorption in their removal is of minor importance.
377

3.5. Biotransformation Products

Twenty two transformation products were tentatively identified in total with mass accuracy ±5 ppm. The m/z range of the candidate TPs ranged from 132.0567 (TP14) to 245.9536 (TP22). For the majority of the candidates, retention times showed the formation of more polar TPs than the parent compounds. A distinctive time trend (absent in the blank, increasing peak over incubation time) was observed for all candidate TPs. All information about TPs is summarized in Table 1. As identification confidence in HR-MS is sometimes difficult to communicate in an accurately way (Bletsou et al. 2015), in the present work we used the levels of identification confidence proposed by Schymanski et al. (2014). BTR presented the higher degree of biotransformation compared to the other BTRs, as previously reported by Huntscha et al. (2014). Five candidate TPs were found in positive mode (TP1-TP5) and 4 more (TP6-TP9) in negative mode. Hydroxylation was the dominant reaction mechanism followed by oxidation and methylation. Previously reported TPs for BTR (Liu et al., 2011; Huntscha et al., 2014) were among the tentatively identified TPs (TP1-TP7, TP9). In total, five TPs (TP3-TP7) were identified by library spectrum match and the records from the online mass spectra database, MassBank, were reported. Two TPs (TP2 and TP8) were tentatively identified and probable structures were proposed. TP1 (1-OH BTR) was confirmed by a reference standard and for TP9 an unequivocal molecular formula was reported (identification level 1 and 4, respectively; Schymanski et al., 2014). Biotransformation of 4TTR showed 5 candidate TPs (TP10-TP14). Hydroxylation and oxidation were found to be the most probable reaction mechanisms for the formation of the TPs. In positive mode only TP10 ($C_7H_5N_3O_2$) was identified with a tentative structure that is illustrated in Table 1. In negative mode, 4 more TPs were identified. Hydroxylation of the benzene ring was identified for TP14 whereas monohydroxylation of the methyl group were

identified for TP13. Both hydroxylation and oxidation reactions were involved in formation of TP11-TP12. For TP12 the probable structure of 4-COOH BTR was proposed by a library spectrum match (Id. level 2a). 5TTR degradation revealed the formation of 3 candidate TPs (TP15-TP17). TP15 was identified to be 5-COOH BTR by a library spectrum match (Id. level 2a). The tentative structure of TP16 ($C_7H_7N_3O$) corresponds to monohydroxylation, whereas TP17 ($C_7H_7N_3O_2$) corresponds to a dihydroxylation of the benzene ring (ident. level 3). To our knowledge, biodegradation products of XTR has not been studied before, and this is the first report of its biotransformation products. Two candidate TPs (TP18-TP19) were found for XTR and tentative structures were proposed (Id. level 3). TP18 ($C_8H_7N_3O_2$) corresponds to the formation of carboxylic acid XTR, while TP19 ($C_8H_9N_3O$) indicates either the monohydroxylation of a methyl group or monohydroxylation of the benzene ring of XTR, which was detected in both positive and negative ionization mode. CBTR did not show any potential TP according to the screened database either in positive or negative ionization mode. Finally, OHBTH has also not been studied before, and this is the first report of its biotransformation products. Three candidate TPs (TP20-TP22) were identified and tentative structures were proposed for OHBTH (Id. level 3). TP20 of OHBTH ($C_8H_7NO_2S$) indicates methoxylation of the benzene ring, whereas the candidate TPs in negative mode TP21 ($C_7H_5NO_2S$) and TP22 ($C_7H_5NO_5S_2$) correspond to a hydroxylation of the benzene ring followed by the formation of a sulfonic ester in one of the two hydroxyl groups, respectively.

4. Conclusions

HMBBR partially removed all target micropollutants. Co-metabolic biodegradation was the major degradation mechanism. AS and biocarriers contributed to the biodegradation to different

extent. HMBBR performance was similar to a low loaded pure MBBR system and more efficient than AS and MBBR systems operating under the same HRT and organic loading conditions. HMBBR biomass and biomass from traditional AS systems showed no differences on the specific removal rate of target compounds; whereas biomass grown in pure MBBR systems was more efficient. BTR presented more biotransformation products among all target compounds.

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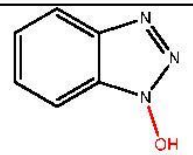
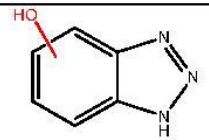
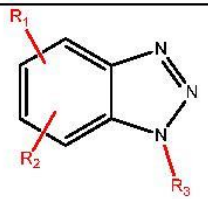
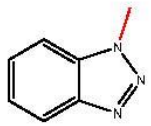
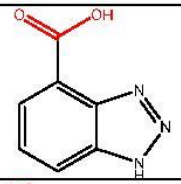
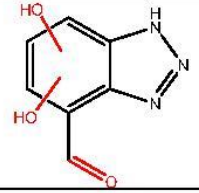
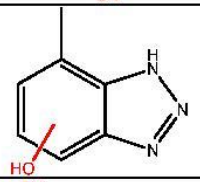
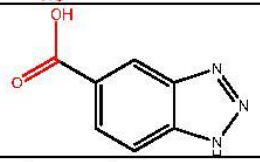
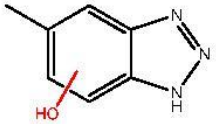
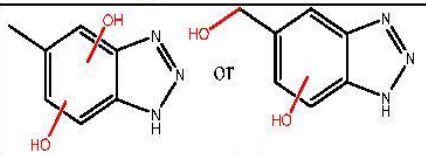
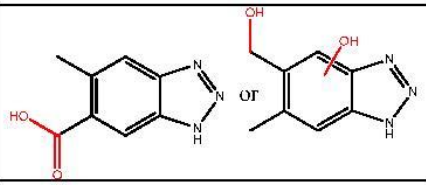
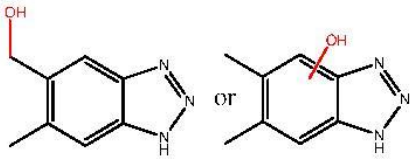
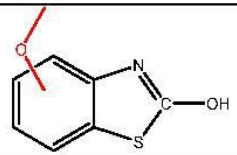
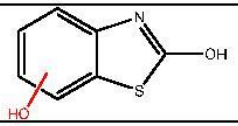
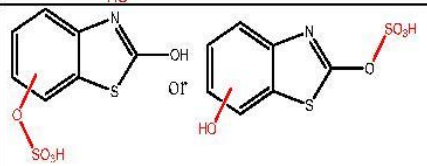
Table 1. Description of candidate TPs observed in batch biodegradation experiments with biomass from HMBBR system

Parent compound	TP	ESI polarity/ Precursor ion	m/z	Rt(min)	Molecular Formula	Tentative Structures	Id. Level (MassBank Record)	Time trend[a]	Reported in Literature
BTR	TP1	$[M+H]^+$	136.0505	3.8	$C_6H_5N_3O$		1	↗	Huntscha et al., 2014
	TP2	$[M+H]^+$	136.0505	4.1	$C_6H_5N_3O$		3	↗	Huntscha et al., 2014
		$[M-H]^-$	134.0360	4.0				↗	
	TP3	$[M+H]^+$	150.0662	5.1	$C_7H_7N_3O$		3 (ETS00101)	↗	Huntscha et al., 2014 Liu et al., 2011
	TP4	$[M+H]^+$	178.0611	3.5	$C_8H_7N_3O_2$	 R_1, R_2, R_3: H, CH_3, COOH	3 (ETS00108)	↗	Huntscha et al., 2014
	TP5	$[M+H]^+$	178.0611	4.2			3 (ETS00109)	↗	Huntscha et al., 2014
	TP6	$[M-H]^-$	132.0567	3.7	$C_7H_7N_3$		2a (ETS00115)	↗	Huntscha et al., 2014 Liu et al., 2011

	TP7	$[M-H]^-$	150.0309	1.6	$C_6H_5N_3O_2$		3 (ETS00103)	↗	Huntscha et al., 2014
	TP8	$[M-H]^-$	150.0309	3.1	$C_6H_5N_3O_2$		3	↗	
	TP9	$[M-H]^-$	182.0207	1.2	$C_6H_5N_3O_4$	-	4	↗	Huntscha et al., 2014
4TTR	TP10	$[M+H]^+$	164.0455	4.1	$C_7H_5N_3O_2$		3	↗	Huntscha et al., 2014
		$[M-H]^-$	162.0309	3.2					
	TP11	$[M-H]^-$	162.0309	2.3	$C_7H_5N_3O_2$		2a (ETS00107)	↗	Huntscha et al., 2014
	TP12	$[M-H]^-$	178.0258	1.3	$C_7H_5N_3O_3$		3	↗	

	TP13	$[M-H]^-$	148.0516	3.9	$C_7H_7N_3O$		3 (ETS00102)	↗↘	Huntscha et al., 2014
	TP14	$[M-H]^-$	148.0516	4.7	$C_7H_7N_3O$		3 (ETS00102)	↗↘	Huntscha et al., 2014
5TTR	TP15	$[M+H]^+$	164.0455	3.7	$C_7H_5N_3O_2$		2a (ETS00121)	↗	Huntscha et al., 2014
		$[M-H]^-$	162.0309	3.7					
	TP16	$[M+H]^+$	150.0662	4.6	$C_7H_7N_3O$		3 (ETS00102)	↗	Huntscha et al., 2014
	TP17	$[M-H]^-$	164.0466	2.9	$C_7H_7N_3O_2$		3	↗	
XTR	TP18	$[M+H]^+$	178.0611	3.8	$C_8H_7N_3O_2$		3	↗	

	TP19	$[M+H]^+$	164.0818	5.4	$C_8H_9N_3O$		3	↗	
		$[M-H]^-$	162.0673	4.9					
CBTR			-						
OHBTH	TP20	$[M+H]^+$	182.0270	3.1	$C_8H_7NO_2S$		3	↗	
	TP21	$[M-H]^-$	165.9968	5.8	$C_7H_5NO_2S$		3	↗	
	TP22	$[M-H]^-$	245.9536	4.2	$C_7H_5NO_5S_2$		3	↗↘	

[a]The symbols(↗) and (↘) in time trend column indicate whether there is an increase or decrease in formation of a specific TP. In red it is indicated the transformation of the parent compound.

Figure 1

Figure 1: Schematic representation, operational characteristics and performance of the HMBBR system used in this study (HRT was equal to 12.4 ± 0.6 h for each reactor; sampling points are indicated with an S).

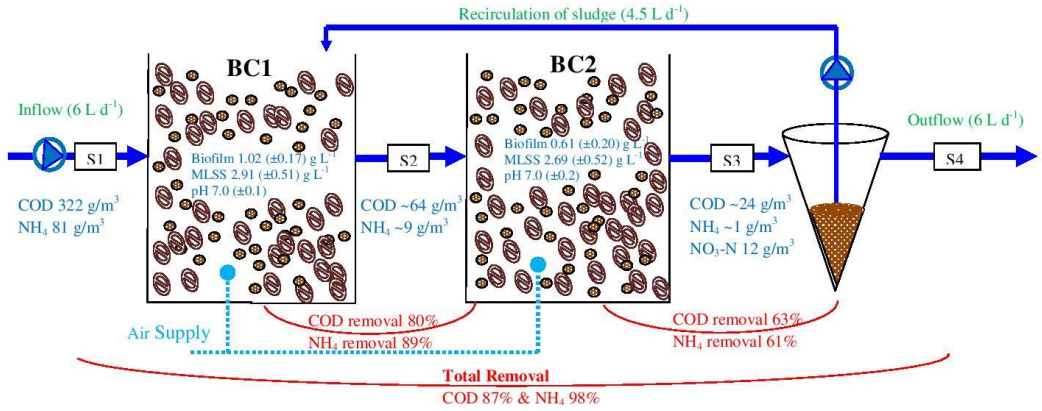


Figure 2

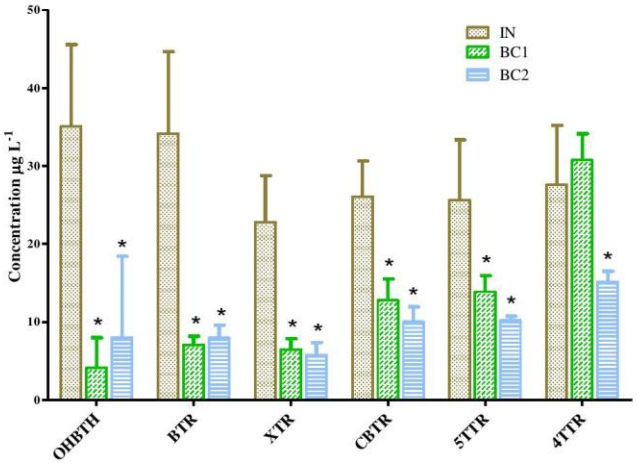


Figure 2: Concentrations (as µg L^{-1}) of target compounds in raw wastewater (IN), effluent wastewater of the 1st bioreactor (BC1) and effluent wastewater of the 2nd bioreactor (BC2) of the HMBBR system (t-bars represent 95% confidence interval; the use of star indicates statistical differences at 95% confidence level from IN sample).

Figure 3

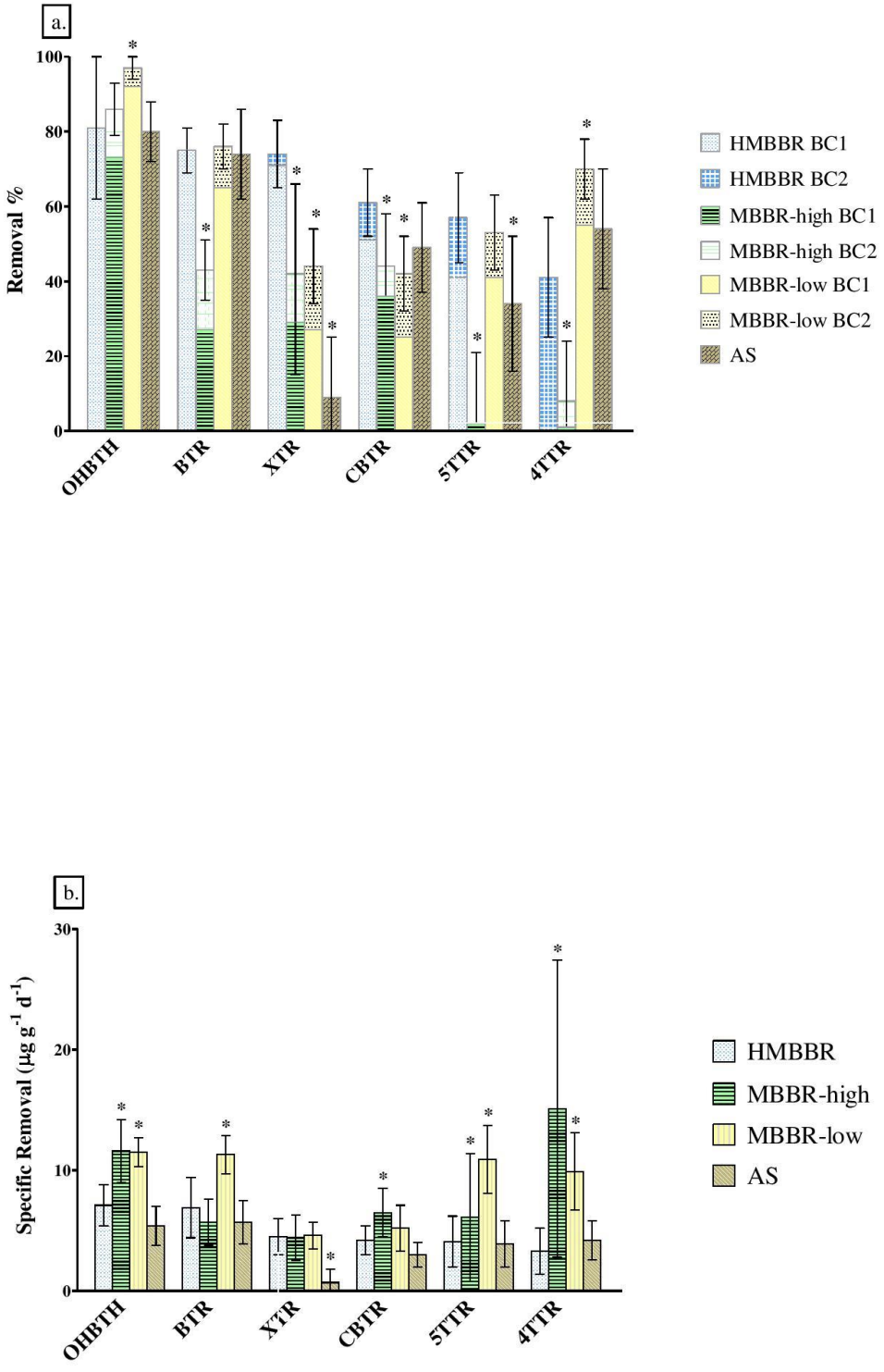


Figure 3: Comparison of the removal efficiency of target compounds (a) and the specific removal of micropollutants (b) in the HMBBR system used in this study with other MBBR and AS systems previously used by Mazioti et al., (2015b). MBBR-high system consisted of two bioreactors in series receiving an organic loading of 0.60 kg m^{-3} d^{-1} and 0.17 kg m^{-3} d^{-1}, respectively; MBBR-low system consisted of two bioreactors in series receiving an organic loading of 0.25 kg m^{-3} d^{-1} and 0.05 kg m^{-3} d^{-1}, respectively and AS operated on an organic loading of 0.25 kg m^{-3} d^{-1} (t-bars represent 95% confidence interval; the use of star indicates statistical differences at 95% confidence level from HMBBR system)

Figure 4

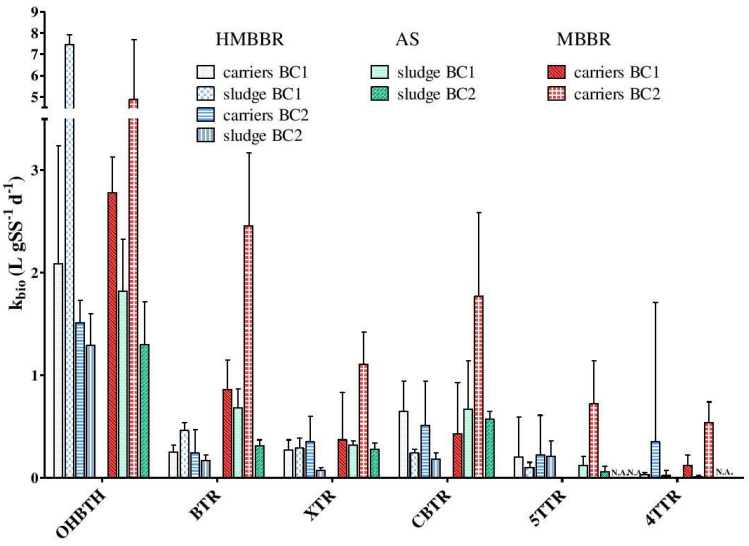


Figure 4: Biodegradation constants (k_{bio}, as L g_{SS}^{-1} d^{-1}) for the HMBBR system calculated in batch experiments with activated sludge and attached biomass from BC1 and BC2, compared with constants from a pure MBBR and a conventional AS system (Mazioti et al., 2015b).

Figure 5

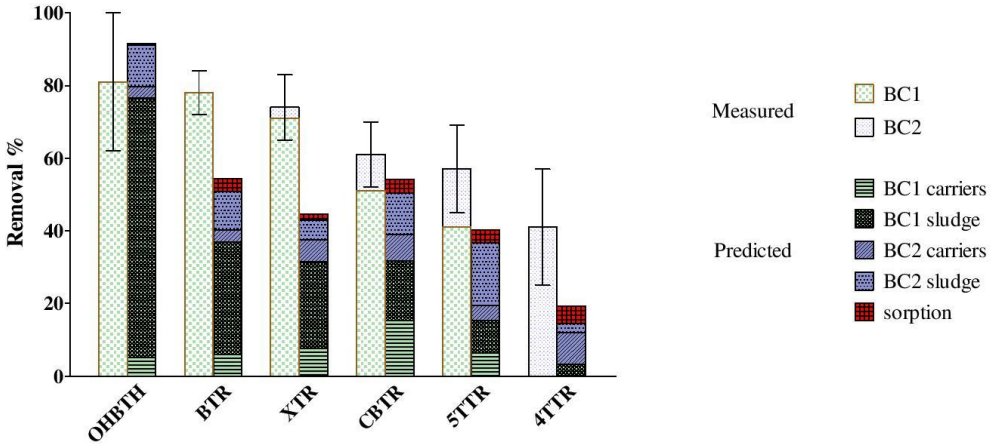


Figure 5: Measured and predicted removal of target compounds in HMBBR system. The contribution of different types of biomass (carriers and sludge) and different mechanisms on their removal is also shown (for predicted removal, the biodegradation with BC1 and BC2 carriers and sludge as well as the sorption on sludge were determined).

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Table S1. Characteristics of raw and treated wastewater in HMBBR system used in this study (n = 10, standard deviations are given in parentheses).

Parameter	Raw wastewater	Treated wastewater
pH	7.0 (±0.4)	7.0 (±0.2)
COD_{dis} (mg L^{-1})	322 (+193)	24 (+14)
NH_4-N (mg L^{-1})	81 (±35)	1.1 (±1.1)
NO_3-N (mg L^{-1})	5.1 (⊥4.0)	12.3 (⊥9.2)
TSS (mg L^{-1})	76 (±66)	35 (±19)

Table S2: Initial conditions applied in batch biodegradation experiments with different types of biomass from bioreactors BC1 and BC2.

Parameter	BC1 carriers	BC1 sludge	BC2 carriers	BC2 sludge
pH	7.02	7.18	7.04	7.22
TSS (mg L^{-1})	1158	3382	776	3739
NH_4-N (mg L^{-1})	53	55	8.5	9.7
NO_3-N (mg L^{-1})	2.5	1.8	7.4	6.8
COD_{dis} (mg L^{-1})	203	223	28	59

Table S3: Elution program concerning the analysis of samples for the determination of transformation products (TPs). The gradient program starts with 1% B constant for 1 min and it increases to 39 % in 2 min, and then to 99.9 % in the following 11 min. Then it keeps constant for 2 min and finally initial conditions were restored within 0.1 min. Gradient was also applied in the flow rate, starting with 0.2 mL min^{-1} for 1 min, increasing to 0.4 mL min^{-1} in 13 min and to 0.48mL min^{-1} in 2 min. Then it keeps constant for 3 min and then the initial flow rate is restored.

Reverse Phase Chromatography			
Time(min)	**Flow rate (mL/min)**	**%A***	**%B***
0.0	0.200	1.0	99.0
0.1	0.200	1.0	99.0
1.0	0.200		99.0
3.0		39.0	61.0
14.0	0.400	99.9	0.1
16.0	0.480	99.9	0.1
16.1	0.480	1.0	99.0
19.0	0.480	1.0	99.0
19.1	0.200	1.0	99.0
20	0.2	1.0	99.0

*Methanol (solvent A) and water:methanol (90:10) (solvent B)

Table S4: Biodegradation constants calculated during batch experiments with biocarriers and activated sludge (AS) from 1st bioreactor (BC1) and 2nd bioreactor (BC2) in HMBBR system (average values and standard deviation), from **Paper C**.

Biodegradation rate constant, k (d^{-1})

verage	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2
	OHBTH			**BTR**			**XTR**			**CBTR**			**5TTR**			**4TTR**	
2.43	1.34	0.902	**0.29**	0.08	0.971	**0.31**	0.11	0.950	**0.75**	0.34	0.935	**0.23**	0.45	0.392		N.A.	
25.22	1.57	0.985	**1.54**	0.26	0.984	**0.98**	0.33	0.925	**0.81**	0.13	0.991	**0.34**	0.17	0.914	**0.09**	0.06	0.669
1.17	0.17	0.985	**0.19**	0.18	0.742	**0.27**	0.20	0.637	**0.40**	0.33	0.774	**0.17**	0.30	0.421	**0.27**	1.05	0.735
4.84	1.17	0.997	**0.63**	0.20	0.916	**0.26**	0.12	0.921	**0.68**	0.23	0.959	**0.79**	0.57	0.841	**0.08**	0.17	0.897

Pseudo first-order biodegradation rate constant, k_{bio} (L g_{ss}^{-1} d^{-1})

verage	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2	average	st.dev.	R^2
	OHBTH			**BTR**			**XTR**			**CBTR**			**5TTR**			**4TTR**	
2.09	1.15	0.902	**0.25**	0.07	0.971	**0.27**	0.10	0.950	**0.65**	0.29	0.935	**0.20**	0.39	0.392		N.A.	
7.46	0.46	0.985	**0.46**	0.08	0.984	**0.29**	0.10	0.925	**0.24**	0.04	0.991	**0.10**	0.05	0.914	**0.03**	0.02	0.669
1.51	0.22	0.985	**0.24**	0.23	0.742	**0.35**	0.25	0.637	**0.51**	0.43	0.774	**0.22**	0.39	0.421	**0.35**	1.36	0.735
1.29	0.31	0.997	**0.17**	0.05	0.916	**0.07**	0.03	0.921	**0.18**	0.06	0.959	**0.21**	0.15	0.841	**0.02**	0.05	0.897

[1]Experiments with biocarriers from BC1 were conducted with COD initial concentration of 203 mg L^{-1}; [2]Experiments with AS from BC1 were conducted with COD initial concentration of 223 mg L-1; [3]Experiments with biocarriers from BC2 were conducted with COD initial concentration of 28 mg L^{-1}; [4]Experiments with AS from BC2 were conducted with COD initial concentration of 59 mg L^{-1}.