University of the Aegean

Department of Environment

PhD Dissertation

"Study on the fate of antimicrobial compounds in *Lemna minor* constructed wetland systems and investigation of the characteristics of the produced biomass"

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Πανεπιστήμιο Αιγαίου

Τμήμα Περιβάλλοντος

Διδακτορική Διατριβή

"Μελέτη της τύχης αντιμικροβιακών ουσιών σε συστήματα τεχνητών υγροτόπων με *Lemna minor* και διερεύνηση των χαρακτηριστικών της παραγόμενης βιομάζας"

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ΦΕΒΡΟΥΑΡΙΟΣ 2021

“Reach what you cannot!”

Report to Greco, Nikos Kazantzakis

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Η Επταμελής Εξεταστική Επιτροπή, η οποία ορίστηκε στην υπ' αριθμ. 05/24.02.2021 Συνεδρίαση της Συνέλευσης του Τμήματος Περιβάλλοντος για την τελική αξιολόγηση και κρίση της Διδακτορικής Διατριβής της Υποψήφιας Διδάκτορος κυρίας Ευαγγελίας Ιατρού και αποτελείται από τους κ.κ.

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συνήλθε σε συνεδρίαση σήμερα 13.04.2021, ημέρα Τρίτη και ώρα 14:00 μ.μ μέσω της υπηρεσίας epresence (σύμφωνα με την υπ' αριθμ. 17/13.03.2020 απόφαση της έκτακτης συνεδρίασης της Συγκλήτου θέμα 2.1 «Συνεδριάσεις Συλλογικών Οργάνων Διοίκησης, Επιτροπών και Συμβουλίων στο πλαίσιο των κατεπειγόντων μέτρων αντιμετώπισης των αρνητικών συνεπειών εμφάνισης του κορωνοϊού COVID-19»), προκειμένου να παρακολουθήσει τη δημόσια υποστήριξη της Διδακτορικής Διατριβής της ανωτέρω υποψήφιας με θέμα «*Study on the fate of antimicrobial compounds in Lemna minor constructed wetland systems and investigation of the characteristics of the produced biomass*» (ελληνικός τίτλος: *Μελέτη της τύχης αντιμικροβιακών ουσιών σε συστήματα τεχνητών υγροτόπων με Lemna minor και διερεύνηση των χαρακτηριστικών της παραγόμενης βιομάζας*).

Μετά την υποστήριξη της Διατριβής από την κυρία Ευαγγελία Ιατρού, σε κλειστή συνεδρίαση την ίδια ημέρα, τα Μέλη της Εξεταστικής Επιτροπής έκριναν ομόφωνα ότι το περιεχόμενο της Διατριβής είναι πρωτότυπο, συμβάλει ουσιαστικά στην πρόοδο της επιστήμης, η παρουσίαση από την Υποψήφια ήταν Άριστη και απέδωσε ομόφωνα τον χαρακτηρισμό Άριστα.

ΠΡΑΚΤΙΚΟ ΑΞΙΟΛΟΓΗΣΗΣ ΤΗΣ ΔΙΔΑΚΤΟΡΙΚΗΣ ΔΙΑΤΡΙΒΗΣ

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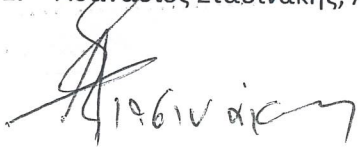
1. Αθανάσιος Στασινάκης, Αναπληρωτής Καθηγητής, Επιβλέπων



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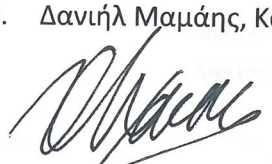

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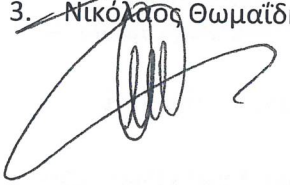

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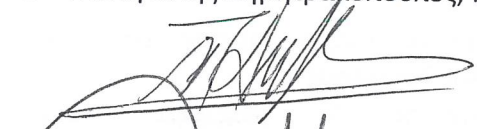

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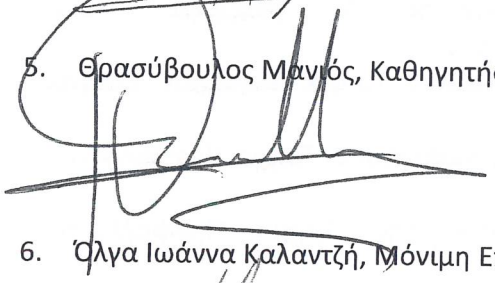

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Abstract

The release of antimicrobials into the environment is a matter of important concern and constitutes a potential threatfor humans' health as well as terrestrial and aquatic ecosystems. Antimicrobials are partially removed during conventional primary and secondary wastewater treatment and as a result they are often detected in the aquatic environment. Since 2000, they have been detected at ppb or pptevels in a variety of environmental media across the globe.

Amongst different plant-based systems used for wastewater treatment, ponds with the duckweed *Lemna minor* have been applied successfully in several countries for the removal of organic matter, nutrients and heavy metals. As this macrophyte is characterized by the high protein or starch content, during the last decade, several studies are also available investigating its cultivation for animals' feedstock or biofuels' production. On the other hand, limited information is available for the ability of such systems to remove organic micropollutants and especially antimicrobial compounds.

The main goals of the current PhD Thesis were: a) to estimate the potential environmental risks associated with human consumption of antimicrobials in Greece, b) to study the growth and characteristics of *L. minor* in human urine and municipal wastewater and c) to investigate the removal efficiency of antimicrobials in *L. minor systems* as well as the role of different abiotic and biotic mechanisms on their elimination.

In the first part of the current study, consumption data were collected for the 24 most often used antimicrobials in Greece for years 2008-2010 and their Predicted Environmental Concentrations (PECs) in raw and treated wastewater were calculated using mass balances. The ecotoxicological risk was estimated by calculating the ratio of PEC to Predicted No Effect Concentration (PNEC) for three categories of aquatic organisms (algae, daphnids and fish). Based on acute toxicity data for algae, an ecological threat seems possible for 7 out of 24 target antimicrobials in raw and treated wastewater, while no significant risk was estimated for daphnids and fish. For Greek rivers where low and medium dilution of wastewater occurs, a moderate to high risk is expected due to the existence of individual antimicrobials such as amoxicillin, clarithromycin, ciprofloxacin, azithromycin, erythromycin and levofloxacin in discharged treated wastewater.

In the second part of the PhD Thesis, experiments were conducted to study the cultivation of duckweed *L. minor* in human urine (HU) and the role of different parameters such as urine

type, dilution factor, temperature, existence of macro- and microelements on growth rate was investigated. The simultaneous removal of nutrients and selected antimicrobials was also studied in experiments with HU and treated domestic wastewater, while the starch and protein content of the produced biomass was determined. Higher growth rates were observed at 24 ^{o}C, using HU stored for 1 d and with dilution factor equal to 1:200. In experiments with HU and wastewater, the removal of COD, total phosphorus and total nitrogen exceeded 80%, 90% and 50%, respectively, while ciprofloxacin and sulfamethoxazole were eliminated by more than 80%. The main removal mechanism for the former antimicrobial was photodegradation, while plant uptake and biodegradation seem to be of significant importance for the latter. Crude protein content reached 31.6% in experiments with HU and biomass harvesting, while starch content was enhanced when duckweed was transferred to water for 21 d, reaching 47.1%.

In the third part of the PhD Thesis, the use of duckweed-based wastewater treatment systems for producing biomass with high crude protein and starch content was investigated. Three lab-scale systems were used; System 1 was planted with *L. minor*, System 2 with *L.gibba* and System 3 with the combination of the two duckweeds. The studied duckweeds were cultivated using secondary treated wastewater as substrate (Phase A), in the presence of excess NH_4-N (Phase B) and using water with no nutrients (Phase C). All systems achieved average NH_4-N removal higher that 90%. The specific duckweeds growth rates and the specific duckweeds growth rates normalized to the area ranged between 0.14 d^{-1} and 8.9 g m^{-2} d^{-1} (System 1) to 0.19 d^{-1} and 14.9 g m^{-2} d^{-1} (System 3). The addition of NH_4-N resulted to an important increase of biomass protein content, reaching 44.4% in System 3, 41.9% in System 2 and 39.4% in System 1. The transfer of biomass in water containing no nutrients resulted to the gradual increment of the starch content up to the end of the experiment. The highest starch content was achieved for the combination of the two duckweeds (46.1%), followed by *L. gibba* (44.9%) and *L. minor* (43.9%).

In the last experimental part of the current PhD Thesis, the fate of four antimicrobials (cefadroxil, CFD; metronidazole, METRO; trimethoprim, TRI; sulfamethoxazole, SMX) was studied in *L. minor* systems and the role of different mechanisms on their removal was evaluated. All micropollutants were significantly removed in batch experiments with active *L. minor* and the order from the highest to lower removal was CFD >METRO > SMX > TRI till the end of experiment. Calculation of kinetic constants for hydrolysis, photodegradation, sorption to biomass and plant uptake revealed significant differences depending on the

compound and the studied mechanism. For METRO, TRI and SMX the kinetic constants of plant uptake were by far higher comparing to those of the other mechanisms. The transformation productsofantimicrobialswere identified using UHPLC-QToF-MS. Two were the main degradation pathways for TRI; hydroxylation takes place during both phyto- and photodegradation, while demethylation occurs only in absence of *L. minor*. The operation of a continuous-flow duckweed system showed METRO and TRI removal equal to 71±11% and 61±8%, respectively. The plant uptake and biodegradation were the major mechanisms for METRO removal while the most important mechanism for TRI was plant uptake.

The structure of the PhD Thesis is the following: Chapter 1 includes a short literature review on the main wastewater treatment processes used in this study and the examined antimicrobials. Information is also provided for theobjectivesand the outline of the Thesis. In Chapter 2, the experimental procedures and analytical methods are described. In Chapter 3, the results that came out of this study are presented in four sub-chapters which are directly related to the four (4) scientific publications in journals produced from this Thesis. Chapter 4 summarizes the most important conclusions and contains suggestions for future research. In the last two chapters the references and the supplementary material (tables and figures) are provided as they came out from the Thesis manuscript and the corresponding publications.

Keywords

Antimicrobials, fate, removal, risk assessment, human urine, municipal wastewater, duckweeds, *Lemna minor*, *Lemna gibba*, constructed wetlands, biomass production, valorization

Περίληψη

Η απελευθέρωση αντιμικροβιακών ουσιών στο περιβάλλον αποτελεί ένα ζήτημα σημαντικού ενδιαφέροντος για την ανθρώπινη υγεία αλλά και την προστασία του περιβάλλοντος. Οι αντιμικροβιακές ουσίες απομακρύνονται συνήθως σε μικρό ποσοστό κατά τη συμβατική επεξεργασία των λυμάτων και κατά συνέπεια συχνά ανιχνεύονται πολύ συχνά στο υδάτινο περιβάλλον. Από το 2000, οι συγκεκριμένες ουσίες έχουν εντοπιστεί σε συγκεντρώσεις της τάξης των ng L^{-1} ή μg L^{-1} σε μια ποικιλία περιβαλλοντικών μέσων ανά την υφήλιο.

Μεταξύ των διαφόρων συστημάτων τεχνητών υγροτόπων που χρησιμοποιούνται για την επεξεργασία των υγρών αποβλήτων, οι λίμνες που περιέχουν το μακρόφυτο *Lemna minor* έχουν χρησιμοποιηθεί με επιτυχία σε διάφορες χώρες για την απομάκρυνση του οργανικού υλικού, των θρεπτικών και των βαρεών μετάλλων. Καθώς αυτό το φυτό χαρακτηρίζεται από υψηλό πρωτεινικό περιεχόμενο και μεγάλη περιεκτικότητα σε άμυλο, κατά την τελευταία δεκαετία, διάφορες ερευνητικές εργασίες είναι διαθέσιμες που μελετούν την καλλιέργειά του για την παραγωγή ζωοτροφών ή βιοκαυσίμων. Από την άλλη πλευρά, μέχρι σήμερα ελάχιστη πληροφορία είναι διαθέσιμη για την ικανότητα αυτών των συστημάτων να απομακρύνουν οργανικούς μικρορρύπους και ειδικότερα αντιμικροβιακές ενώσεις.

Οι κύριοι στόχοι της παρούσας εργασίας ήταν α) να εκτιμηθούν οι περιβαλλοντικοί κίνδυνοι που συνδέονται με την ανθρώπινη κατανάλωση αντιμικροβιακών ουσιών στην Ελλάδα, β) να μελετηθεί η ανάπτυξη και τα χαρακτηριστικά της *L. minor* σε ανθρώπινα ούρα και αστικά υγρά απόβλητα και γ) να διερευνηθεί η απομάκρυνση των αντιμικροβιακών ουσιών σε συστήματα *L. minor* καθώς επίσης και ο ρόλος των αβιοτικών και βιοτικών μηχανισμών στην απομάκρυνσή τους.

Κατά την πρώτη φάση της διδακτορικής διατριβής, πραγματοποιήθηκε συλλογή δεδομένων για την κατανάλωση σκευασμάτων που περιέχουν αντιμικροβιακές ουσίες στην Ελλάδα και εκτιμήθηκαν οι αναμενόμενες περιβαλλοντικές συγκεντρώσεις (predicted environmental concentration, PEC) στα υγρά απόβλητα χρησιμοποιώντας ισοζύγια μάζας. Έπειτα έγινε συλλογή δεδομένων οξείας τοξικότητας, είτε βιβλιογραφικά, είτε μέσω μοντέλων εκτίμησης οξείας τοξικότητας για τρεις διαφορετικές κατηγορίες υδρόβιων οργανισμών (άλγη, δαφνίδες, ψάρια) με σκοπό να υπολογιστουν οι προβλεπόμενες συγκέντρωσεις που δεν προκαλουν επιπτώσεις (predicted no-effect concentration, PNEC). Τέλος, βάσει των παραπάνω δεδομένων εκτιμήθηκε το πηλίκο επικινδυνότητας (risk quotient, RQ) στο υδάτινο

περιβάλλον για κάθε έναν οργανισμό ξεχωριστά. Βασιζόμενοι στα δεδομένα οξείας τοξικότητας, πιθανός οικολογικός κίνδυνος φαίνεται για 7 από τις 24 αντιμικροβιακές ουσίες που μελετήθηκαν για τα ανεπεξέργαστα και τα επεξεργασμένα αστικά λύματα, ενώ δεν αναμένεται κάποιος κίνδυνος για τις δαφνίσες και τα ψάρια. Για τα ελληνικά ποτάμια, όπου παρατηρείται μικρή ή μεσαία αραίωση των αποβλήτων, αναμένεται μέσος έως υψηλός κίνδυνος εξαιτίας της παρουσίας των ουσιών amoxicillin, clarithromycin, ciprofloxacin, azithromycin, erythromycin και levofloxacin.

Κατά τη δεύτερη φάση της διδακτορικής διατριβής, πραγματοποιήθηκαν πειράματα για να μελετηθεί η καλλιέργεια του μακρόφυτου *L. minor* σε ανθρώπινα ούρα (HU) να εξεταστεί ο ρόλος διαφορετικών παραμέτρων όπως του τύπου των ούρων, του συντελεστή αραίωσης, της θερμοκρασίας και της ύπαρξης μάκρο- και μίκροθρεπτικών στην ταχύτητα ανάπτυξής του. Μελετήθηκε επίσης η ταυτόχρονη απομάκρυνση θρεπτικών και επιλεγμένων αντιμικροβιακών ενώσεων σε πειράματα με ούρα και επεξεργασμένα αστικά λύματα, ενώ διερευνήθηκε το περιεχόμενο της βιομάζας σε άμυλο και πρωτείνες. Υψηλές ταχύτητες αύξησης παρατηρήθηκαν στους 24 ^{o}C, χρησιμοποιώντας ούρα αποθηκευμένα για 1 ημέρα και συντελεστή αραίωσης 1:200. Στα πειράματα με ούρα και απόβλητα, η απομάκρυνση του COD, του ολικού φωσφόρου και του ολικού αζώτου ξεπέρασε το 80%, 90% και 50%, αντίστοιχα, ενώ το ciprofloxacin και το sulfamethoxazole απομακρύνθηκαν κατά περισσότερο από 80%. Ο κύριος μηχανισμός απομάκρυνσης της πρώτης ουσίας ήταν η φωτοδιάσπαση, ενώ της δεύτερης η πρόσληψη από τα φυτά και η βιοαποδόμηση. Το πρωτεινικό περιεχόμενο της βιομάζας έφθασε το 31.6% στα πειράματα με τα ούρα, ενώ το περιεχόμενο σε άμυλο αυξήθηκε όταν το φυτό μεταφέρθηκε σε νερό για 21 ημέρες, φθάνοντας το 47.1%.

Στην τρίτη πειραματική φάση, πραγματοποιήθηκαν πειράματα με τη χρήση επεξεργασμένων αποβλήτων και δυο φωτοσυνθετικών οργανισμών που ανήκουν στα duckweeds για παραγωγή βιομάζας με υψηλό περιεχόμενο σε πρωτείνες και άμυλο. Χρησιμοποιήθηκαν 3 πειραματικές διατάξεις; το Σύστημα 1 περιείχε *L.minor,* το Σύστημα 2 *L.gibba* και το Σύστημα 3 συνδυασμό των παραπάνω οργανισμών. Τα μελετώμενα είδη καλλιεργήθηκαν σε δευτεροβάθμια επεξεργασμένα υγρά απόβλητα (Φάση Α), παρουσία επιπλέον NH_4-N (Φάση Β) και παρουσία νερού χωρίς θρεπτικά (Φάση Γ). Όλα τα συστήματα πέτυχαν μέση απομάκρυνση αμμωνιακού αζώτου μεγαλύτερη του 90%. Η ειδική ταχύτητα αύξηση των φυτών κυμάνθηκε μεταξύ 0.14 d^{-1} (Σύστημα 1) και 0.19 d^{-1} (Σύστημα 3). Η προσθήκη NH_4-N συνετέλεσε σε σημαντική αύξηση του πρωτεινικού περιεχομένου που έφθασετο 44.4% στο

Σύστημα 3, 41.9% στο Σύστημα 2 και 39.4% στο Σύστημα 1. Η μεταφορά της βιομαζας στο νερό που δεν περιείχε θρεπτικά οδήγησε σε βαθμιαία αύξηση της περιεκτικότητας σε άμυλο. Η μεγαλύτερη συγκέντρωση σε άμυλο παρατηρήθηκε για το συνδυασμό των δύο μακροφύτων (46.1%), ακολουθούμενη από τη *L. gibba* (44.9%) και τη *L. minor* (43.9%).

Στο τελευταίο πειραματικό στάδιο της παρούσας διατριβής, μελετήθηκαν οι μηχανισμοί της υδρόλυσης, φωτοδιάσπασης, προσρόφησης, πτητικοποίησης και η μικροβιακή βιοαποδόμηση τεσσάρων αντιμικροβιακών ουσιών (cefadroxil, CFD; metronidazole, METRO; trimethoprim, TRI; sulfamethoxazole, SMX) σε συστήματα ασυνεχούς τροφοδοσίας που περιείχαν τον οργανισμό *L. minor*. Όλες οι εξεταζόμενες ουσίες απομακρύνθηκαν σημαντικά στα πειράματα ασυνεχούς τροφοδοσίας, μεγαλύτερη απομάκρυνσηέως το τέλος του πειράματος -κατά φθίνουσα σειρά- παρατηρήθηκε για τις ουσίες CFD>METRO>SMX>TRI. Υπολογισμός των κινητικών σταθερών για την υδρόλυση, φωτοδιάσπαση, ρόφηση στη βιομάζα και πρόσληψη από τα φυτά έδειξε σημαντικές διαφοροποιήσεις ανάλογα με την ουσία και τον μελετούμενο μηχανισμό. Παράλληλα αναγνωριστηκαν τα προιόντα μετατροπής των αντιμικροβιακών ενώσεων με χρήση UHPLC-QToF-MS. Για την ουσία trimethoprim βρέθηκε ότι οι δύο βασικοί οδοί αποδόμησής της είναι η υδροξυλίωση που λαμβάνει χώρα τόσο κατά τη φύτο-αποδόμηση όσο και κατά τη φωτοδιάσπαση και η απομεθυλίωσηπου παρατηρείται απουσία της *L. minor*. Η λειτουργία ενός συστήματος συνεχούς ροής με *L. minor* έδειξε ότι οι ουσίες METRO και TRI απομακρύνθηκαν κατά 71±11% και 61±8%, αντίστοιχα. Η πρόσληψη από τα φυτά και η βιοαποδόμηση ήταν οι βασικοί μηχανισμοί απομάκρυνσης του METRO ενώ η πρόσληψη από τα φυτά για τοTRI.

Η δομή της παρούσας διδακτορικής διατριβής είναι η ακόλουθη: Το Κεφάλαιο 1 περιλαμβάνει μια σύντομη βιβλιογραφική ανασκόπηση των μεθόδων επεξεργασίας που χρησιμοποιήθηκαν και τωνυπό μελέτη ουσίες. Παράλληλα, παρουσιάζονται η καινοτομία και οι στόχοι της διδακτορικής διατριβής. Στο Κεφάλαιο 2 παρουσιάζονται εν συντομία όλες οι μεθοδολογίες που ακολουθήθηκαν καθώς και οι αναλυτικές μέθοδοι που χρησιμοποιήθηκαν. Στο Κεφάλαιο 3, παρουσιάζονται τα ερευνητικά αποτελέσματα σε τέσσερα υποκεφάλαια, που συνδέονται άμεσα με τις τέσσερις δημοσιεύσεις σε επιστημονικά περιοδικά που προέκυψαν κατά την εκπόνηση της παρούσας διδακτορικής διατριβής. Στο Κεφάλαιο 4 γίνετε σύνοψη των βασικών συμπερασμάτων καθώς κατατίθενται προτάσεις για μελλοντική έρευνα. Στο Κεφάλαιο 5 βρίσκονται οι επιστημονικές αναφορές που

χρησιμοποιήθηκαν.Τέλος, στο Κεφάλαιο 6 παρατίθενται όλα τα συμπληρωματικά στοιχεία (πίνακες, σχήματα) όπως έχουν προκύψει από κάθε μια επιστημονική δημοσίευση.

Λέξεις κλειδιά

Αντιμικροβιακές ουσίες, τύχη, απομάκρυνση, εκτίμηση κινδύνου, ανθρώπινα ούρα, υγρά απόβλητα,υδροχαρή φυτά, *L.minor*, *L. gibba*, τεχνητοί υγρότοποι, παραγωγή και αξιοποίηση βιομάζας

List of publications

Papers in Scientific Journals

Paper I Evangelia I. Iatrou, Athanasios S .Stasinakis, Nikolaos S. Thomaidis (2014) "Consumption-based approach for predicting environmental risk in Greece due to the presence of antimicrobials in domestic wastewater" Environmental Science and Pollution Research, 21:12941–12950. *DOI: 10.1007/s11356-014-3243-7*

Paper II Evangelia I. Iatrou, Athanasios S. Stasinakis, Maria Aloupi (2015) "Cultivating duckweed *Lemna minor* in urine and treated domestic wastewater for simultaneous biomass production and removal of nutrients and antimicrobials", Ecological Engineering, 84:632-639. *DOI: 10.1016/j.ecoleng.2015.09.071*

Paper III Evangelia I. Iatrou, Elianta Kora, Athanasios S. Stasinakis (2019) "Investigation of biomass production, crude protein and starch content in laboratory wastewater treatment systems planted with *Lemna minor* and *Lemna gibba*" Environmental Technology,40:2649-2656. *DOI:10.1080/09593330.2018.1448002*

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Paper III Evangelia I. Iatrou, Athanasios S. Stasinakis, Nikolaos S. Thomaidis “Fate of Antimicrobial Compounds in Duckweed *Lemna minor* Wastewater Treatment Systems under Batch and Continuous Flow Conditions” SIWW Water Convention June 1-5 2014, International Water Week, Singapore (Ref. No: 2428914) (poster presentation)

Paper IV Evangelia I. Iatrou, Maria Aloupi, Athanasios S. Stasinakis, Nikolaos S. Thomaidis “Growing *Lemna minor* in human and synthetic urine for biomass production, nutrients and antimicrobials removal” 11th International Phytotechnologies Conference, September 30 – October 3, 2014, Heraklion, Crete, Greece, p. 314 (Ref. No: 295) (oral presentation)

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constitutes a great absence in my life. She will always be a part of me and I thank her for her endless love and trust in me since my childhood.

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Some times in your life there are situations that hold you back from your goal but in the end when you know what you want, it is certain that you will succeed. Passing through different situations in and out of my PhD Dissertation, I was taught to focus only to the important parts of life because "*You only live once*"!!!

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List of Abbreviations

ACN: Acetonitrile

AF: Assessment Factor

BOD: Biological Oxygen Demand

CFD: Cefadroxil

CIP: Ciprofloxacin

COD: Chemical Oxygen Demand

DAD: Diode Array Detector

DF: Dilution Factor

DO: Dissolved Oxygen

EC: European Commission

EC_{50}: Median Effect Concentration

EPA: European Protection Agency

ERA: Environmental Risk Assessment

HPLC: High Pressure Liquid Chromatography

HRT: Hydraulic Retention Time

HU: Human Urine

k: First Order Biodegradation Rate Constant

k_{bio}: Biodegradation Rate Constant

K_d: Sorption Coefficient

K_{ow}: Octanol-Water Coefficient

L. minor: *Lemna minor*

L. gibba: *Lemna gibba*

LC50: Median Lethal Concentration

LOD: Limit of Detection

LOEC: Lowest Observed Effect Concentration

LOQ: Limit of Quantification

MEC: Measured Environmental Concentration

MeOH: Methanol

METRO: Metronidazole

NaN_3: Sodium Azide

$NaNO_3$: Sodium Nitrate

NH_4-N: Ammonia-N

NO_3-N: Nitrate-N

NOEC: No Observed Effect Concentration

PEC: Predicted Environmental Concentration

PhCs: Pharmaceuticals

PNEC: Predicted No Effect Concentration

PCPs: Pharmaceuticals and Personal Care Products

QToF-MS: Quadrupole-Time-of-Flight High-Resolution Mass Spectrometer

RQ: Risk Quotient

SIS: Swedish Standard Medium

SMX: Sulfamethoxazole

SPE: Solid Phase Extraction

STP: Sewage Treatment Plant

T: Temperature

TGD: Technical Guidance Document

TN: Total Nitrogen Concentration

TP: Total Phosphorous

TPs: Transformation Products

TR: Toxic Ratio

TRI: Trimethoprim

TSS: Total Suspended Solids

UHPLC: Ultrahigh-Performance Liquid Chromatography

1. Literature Review

1.1 Emerging pollutants – The case of antimicrobial compounds

The focus of environmental research has expanded beyond traditional pollutants into Emerging Pollutants. This is a critical issue in many countries due to their permanent usage and their potential risk in human health and the environment. Pharmaceuticals compounds (PhACs) are emerging pollutants and have been designed in order to cure and treat disease, improve health, and increase life span. They are complex molecules with different functionalities, physico-chemical and biological properties. PhACs can be classified according to their purpose, biological activity and the mode of action (Homem and Santos, 2011). More than 3000 pharmaceutical compounds are used in human medicine in the European Union (EU) and the annual production amount exceeds hundreds of tons (Suza and Feris, 2017; Kümmerer, 2009c; Gros et al., 2010; Kim and Aga, 2007; Bendz et al, 2005). Among all PhACs, “antibiotics” or “antimicrobials” or “anti-infectives” are a significant group and will be discussed further in the following subsections. In the rest of PhD Thesis manuscript, the term antimicrobials or antimicrobial compounds will be used.

1.1.1 Classification of antimicrobials and physicochemical properties

According to the National Organization for Medicines, there are several antimicrobial classes. A widely used classification of antimicrobials is that classifying them into broad spectrum (active in more microbes) and narrow spectrum (active in specific microbes) compounds. The new generation of antimicrobials have a broader spectrum of activity, they have better distribution in the body and are not as susceptible to microbial defenses as the microbes cannot override them (National Organization for Medicines, 2007). As already mentioned, antimicrobial compounds belong to one of the largest classes of pharmaceutical products. After the discovery of penicillin in 1928 by Fleming, the term "antibiotic" was used to characterize only the substances extracted from a fungus or other microorganism, but now also includes all synthetic and semi-synthetic drugs having antibacterial effects.

The antimicrobials are defined as compounds which eliminate or inhibit the growth of other microorganisms. However, the term “antibiotic” extended for antibacterial, antiviral,

antifungal and antitumor activity. Most of these agents are of microbial origin, but may also be semi-synthetic or fully synthetic compounds. There are relatively small molecules and can be classified by either their chemical structure or mechanism of action. The term antimicrobial was used in the manuscript of the current PhD Thesis. All the categories of antimicrobial compounds according to the National Organization for Medicines are listed below in Table 1.1.1:

Table 1.1.1 The categories of antimicrobial compounds according to the National Organization for Medicines

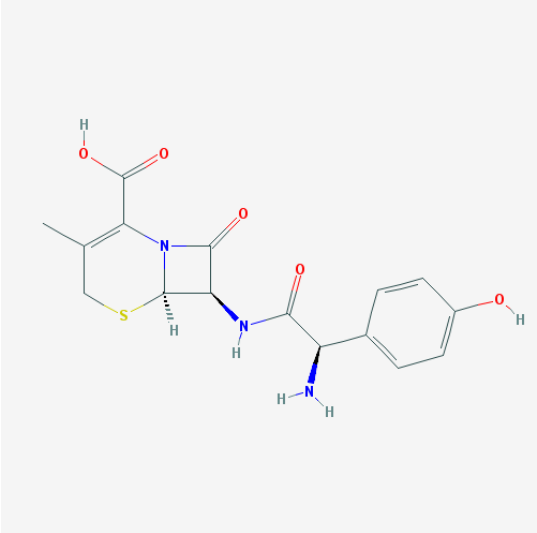
A/A	**Categories of antimicrobials**
1	Penicillin G and Penicillin acid-fast
2	Ampicillin and related beta lactams
3	Inhibitors of beta-lactamases
4	Cephalosporins first generation
5	Cephalosporins second generation
6	Cephalosporins third generation
7	Cephalosporins fourth generation
8	Carbacephem
9	Monobactams
10	Carbapenems
11	Aminoglycosides
12	Macrolides
13	Lincosamides
14	Various other antimicrobials
15	Glycopeptides
16	Tetracyclines
17	Sulfonamides
18	Quinolones – fluoroquinolones
19	Urinary tract antimicrobials
20	Antituberculosis drugs
21	Imidazoles

The antimicrobial compounds that were investigated in the current PhD Thesisare listed below.

Beta-lactams - Subcategory Cephalosporins

All beta-lactams havea ring lactam as their basic structure which is responsible for their antibacterial activity, while their different side chains are the result of different pharmacological properties among the class of substances. Beta-lactams are divided into two categories; penicillins and cephalosporins (Cha et al., 2006; Zhang and Li, 2011). The antimicrobial cefadroxil was investigated in the current PhD Thesis. This compound belongs to the subcategory of cephalosporin.

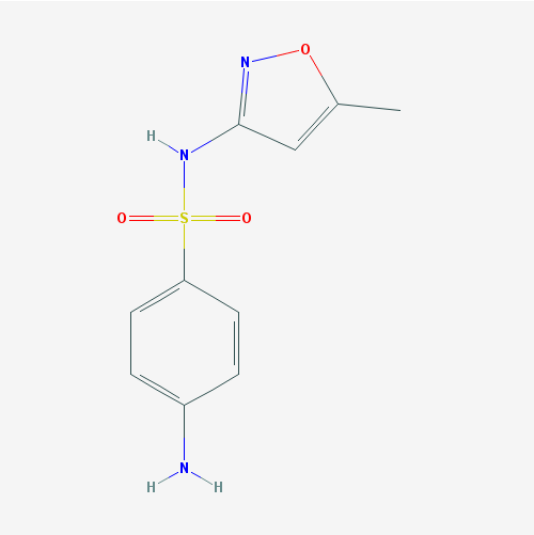
Table 1.1.2 Chemical and physical data of Cefadroxil

Antimicrobial	Cefadroxil **(CFD)**
CAS Number	66592-87-8
Molecular formula	$C_{16}H_{17}N_3O_5S$
Molecular weight	363.389g mol^{-1}
IUPAC Name	(6R,7R)-7-[[(2R)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid
Structural formula	 *Website-source:* *https://pubchem.ncbi.nlm.nih.gov/compound/47965* *(last access 22/11/2016)*

Sulfonamides

Sulfonamides belong to a large class of broad-spectrum antimicrobials which have been used very often since the early 1940s. They count for almost 6% and 12% of the total antimicrobials' consumption in Switzerland and China, respectively (Zhang and Li, 2011). The derivatives of the sulfonamides are applied both in human and veterinary medicine as antibacterial drugs. Their structure corresponds to synthetic antimicrobials containing the group of the sulfonamides. Such substance shall have a free amino group on one side. Various sulfonamides complexes with silver (Ag) or zinc (Zn) have been used as antifungal. They are substances with extensive use and they are prescribed in combination with other synthetic substances such as trimethoprim. From the class of sulfonamides, the substance of sulfamethoxazole was investigated in this Thesis, as it is one of the most often used and holds the highest consumptions in its class (Zhou and Moore, 1997; Baran et al., 2011).

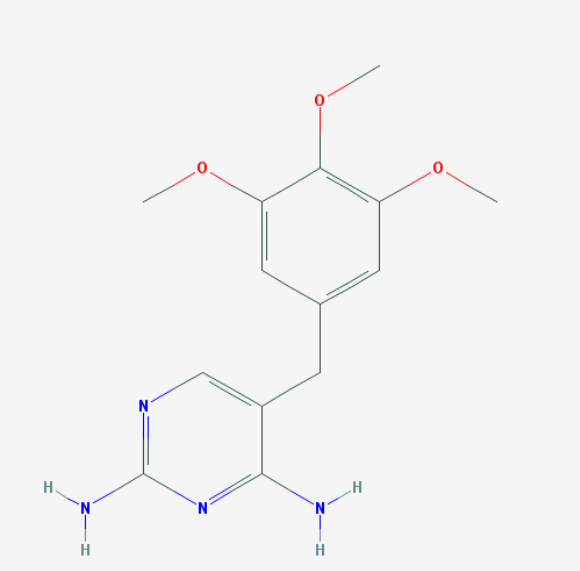
Table 1.1.3 Chemical and physical data of Sulfamethoxazole

Antimicrobial	Sulfamethoxazole **(SMX)**
CAS Number	723-46-6
Molecular formula	$C_{10}H_{11}N_3O_3S$
Molecular weight	253.279g mol^{-1}
IUPAC Name	4-amino-N-(5-methyl-1,2-oxazol-3-yl)benzenesulfonamide
Structural formula	 *Website-source:* *https://pubchem.ncbi.nlm.nih.gov/compound/5329* *(last access 22/11/2016)*

Other antimicrobials

This category contains different types of antimicrobials which in most cases are used in combination with an antimicrobial compound from the other categories. A commonly used compound of this category is trimethoprim which is used almost exclusively with sulfamethoxazole in a fixed ratio of 1:5. The combination of these antibiotics has been used in bronchitis, pneumonia and urinary tract infections (Zhou and Moore, 1997; Zhang and Li, 2011).

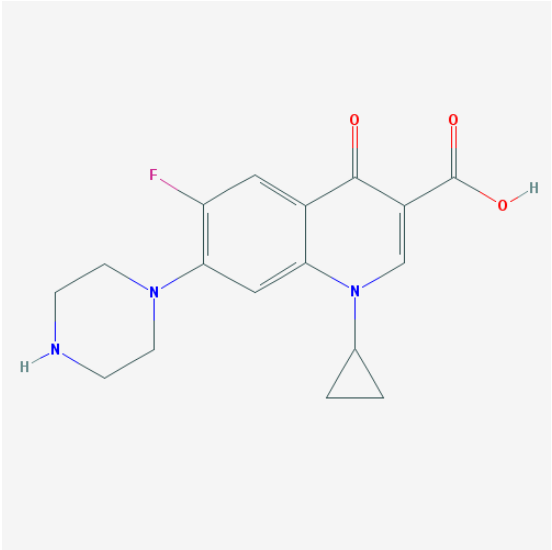
Table 1.1.4 Chemical and physical data of Trimethoprim

Antimicrobial	Trimethoprim **(TRI)**
CAS Number	738-70-5
Molecular formula	$C_{14}H_{18}N_4O_3$
Molecular weight	290.32g mol^{-1}
IUPAC Name	5-[(3,4,5-trimethoxyphenyl)methyl]pyrimidine-2,4-diamine
Structural formula	 *Website-source:* *https://pubchem.ncbi.nlm.nih.gov/compound/5578* *(last access 22/11/2016)*

Quinolones – Subcategory (Fluoroquinolones)

Quinolones belong to a broad spectrum antimicrobials' class where their primary group is quinolone. Fluoroquinolones are a subcategory of quinolones which contain a fluorine atom attached to the central ring. Since the 1960s, where nalidixic acid was discovered, four generations of antimicrobials belonging to the same family have been developed. This category of compounds occupies the fourth position in percentage consumptionofantimicrobials for human use (Park et al., 2002; Xiao et al., 2008; Zhang and Li, 2011). From the class of fluoroquinolones, ciprofloxacin was investigated during the current Thesis.

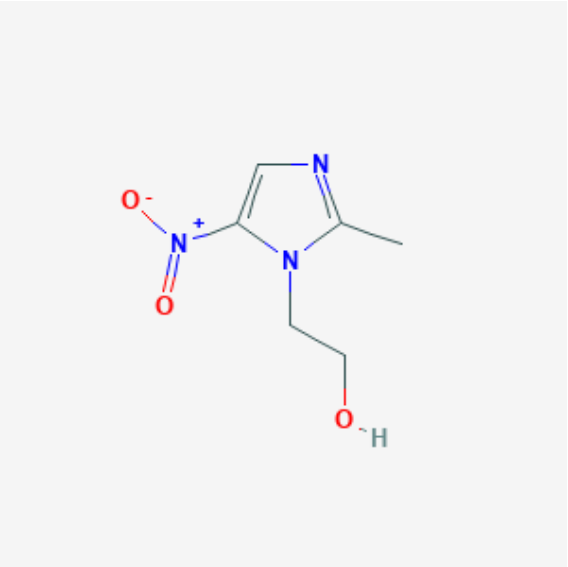
Table 1.1.5 Chemical and physical data of Ciprofloxacin

Antimicrobial	Ciprofloxacin **(CIP)**
CAS Number	085721-33-1
Molecular formula	$C_{17}H_{18}FN_3O_3$
Molecular weight	331.346g mol^{-1}
IUPAC Name	1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid
Structural formula	 *Website-source:* *https://pubchem.ncbi.nlm.nih.gov/compound/2764* *(last access 22/11/2016)*

Imidazole

The substance of this category belongs to the class of nitroimidazoles. Specifically, metronidazole is an antibacterial (for anaerobic bacteria) and antiprotozoal and it is widely used in human and veterinary medicine, including fish farming (Sagan et al., 2005). Metronidazole has been reported for its toxic properties; it is metabolized to several derivatives while its most important metabolites have an alcohol and an acid derivative. Furthermore, its metabolites are carcinogenic and mutagenic in various animal species. The use of metronidazole has been prohibited in the EU, USA and other countries in food-producing species (Voogd, 1981; European Commission, 2005).

Table 1.1.6 Chemical and physical data of Metronidazole

Antimicrobial	Metronidazole **(METRO)**
CAS Number	443-48-1
Molecular formula	$C_6H_9N_3O_3$
Molecular weight	171.156 g mol^{-1}
IUPAC Name	2-(2-methyl-5-nitroimidazol-1-yl)ethanol
Structural formula	 *Website-source:* *https://pubchem.ncbi.nlm.nih.gov/compound/4173#section=Top* *(last access 22/11/2016)*

The physicochemical properties of different antimicrobials vary significantly even if they belong to the same category. The distribution of antimicrobials in the different environmental matrices is affected by their physicochemical properties such as their molecular structure, size, shape, solubility and hydrophobicity (Zhang et al., 2014). The values of octanol-water partition coefficient (K_{ow}), sorption coefficient (K_d), ionization constant (pK_a), Henry law constant determine in which environment compartment (air, water, soil) each compound will be concentrated. Compounds with high K_d values tend to be sorbed to soil materials through the adsorption process, while the pH of the medium and the pKa value of a compound determine its ionized or non-ionized form (Zhang et al., 2014; Cha et al., 2006; Thiele-Bruhn, 2003; Park et al., 2002).

The physicochemical properties of the studied substances are reported in Tables S3.2.1 and S3.4.1 in Chapter 6 (Supplementary and Materials).

1.1.2 Sources and occurrence of antimicrobials in the environment

Antimicrobials have beenextensively used as human and veterinary medicinesto treat microbial infections in humans and animals. They are also used as growth promoters in livestock and poultry. Their environmental occurrence and fate has raised important scientific and public concern during the last 20 years due to theirwidespreaduse and the resulted microbial resistance observed in different environmental compartments (Felis et al., 2020; Kim and Aga, 2007; Kümmerer, 2009 a,b; Escher et al., 2011; Chee-Sanford et al., 2001).

Human and animal antimicrobials enter the environment primarily after excretion from patients/animals through urine and faeces, as unchanged parent molecules or as inactive metabolites (Kümmerer, 2009a; Kim and Aga, 2007; Escher et al., 2011; Bound et al., 2004). Due to the above, the most important sources of antimicrobials to the aquatic environment are hospitals, homes and livestock farms (Christian et al., 2003; Diaz et al., 2003; Kümmerer, 2009a; Le-Minh et al., 2010; Gros et al., 2010; Kim and Aga, 2007; Turkdogan and Yetilmezsoy, 2009). The disposal of unused medicine and the release of these compounds from the pharmaceutical manufacturing processes are also important point-sources. Additionally, their incomplete removal during wastewater treatment, result to the characterization of STPs as constant sources of these compounds in the environment (Felis et

al., 2020; Gros et al., 2010; Kim and Aga, 2007; Li and Zhang, 2010). For example, the concentration of sulfonamide antimicrobials residue, among them and sulfamethoxazole in the environment is low, usually at μg/L level in surface water and wastewater due to the "pseudo-durability" of sulfonamide antimicrobials. Commonly, trimethroprim appears in the same range due to the simultaneous use with sulfamethoxazole (Li and Zhang, 2010).

1.1.3 Toxicity of antimicrobials in aquatic organisms

In February 2006, it was established the Directive 2006/11/EC, which refers to the pollution caused by certain dangerous substances discharged into the EU aquatic environment. The Member States have the obligation to protect the aquatic environment from certain persistent, toxic and bio-accumulative substances. The Directive 2000/60/EC (Water Framework Directive) was established a framework for Community action in the field of water policy, since then the Directive 2013/39/EU was amended and a watch list was established including two pharmaceuticals. Finally, the watch list was adopted with Decision 2015/495 and among other chemicals, three macrolide antimicrobials were added.

With regard to the toxicity of antimicrobials, various toxicity tests have been carried out so far, mainly on aquatic organisms and microorganisms present in the activated sludge. Most of the published papers have focused on acute toxicity, while fewer information is available for the chronic toxicity (Välitaloet al., 2017).

Green and blue algae have been widely used to investigate the toxicity of antimicrobial compounds. For the target antimicrobials of this PhD Thesis, a moderate toxicity has been reported for sulfamethoxazole (EC_{50}: 16.32 μg L^{-1}- 48.86 μg L^{-1}) while the effect of trimethoprim was negligible to green and blue algae (Isidori et al., 2005; Kim et al., 2007; van der Grinten et al., 2010). Ciprofloxacin was highly toxic to cyanobacteria compared to green algae (Robinson et al., 2005; Ebert et al., 2011). Regarding metronidazole, no acute effect has been observed in green algae with an EC50 value of 705 mg L^{-1} (Kołodziejska et al., 2013).

The measurement of toxicity using the marine bacterium *Vibrio fisheri* is a fast and easy way for measuring the toxicity of organic compounds. Among the target compounds, so far, there are studies investigating the toxicity of sulfamethoxazole and ciprofloxacin to this bacterium. Ciprofloxacin presents a relative toxicity with inhibition effects to *Vibrio fisheri* at concentrations up to 5 mg L^{-1} (Hernando et al., 2007). In contrast, to another study, it was

found that in basic pH ciprofloxacin causes no toxic effect on *Vibrio fisher i*in a concentration up to 0.3 mg L^{-1} (Vasconcelos et al., 2009). Regarding sulfamethoxazole, no toxic effects appeared on bacterium *Vibrio fisheri*. However, the by-products of this compound, after the application of Fenton oxidation processes, may be more toxic and may cause toxicity and adverse effects on the bacterium (Klamerth et al., 2010).

Regarding the crustacean aquatic organism *Daphnia magna* and the other organisms belonging to this group, few studies have been published on the toxicity of target antimicrobials. Experiments with the trimethoprim and sulfamethoxazole showed a moderate toxicity in *Daphnia* magna with EC_{50} values ranging between 98.9 μg $L^{-1}$to 196.3 μg $L^{-1}$and 145.6μg $L^{-1}$to 229.5 μg L^{-1}, respectively (Kim et al., 2007; Park and Choi, 2008). Another study with ciprofloxacin showed negligible toxicity to crustaceans, although in another study it has been reported that the fluroquinolones cause acute phytotoxicity in *Daphnia magna* (Kim et al., 2009). In a more recent study, ciprofloxacin showed moderate acute toxicity while it was observed potential chronic effect on *Daphnia magna* reproduction (Martins et al., 2012). Wollenberger et al. (2000) investigated the acute and chronic toxicity of 9 antimicrobials (metronidazole among the studied compounds) and reported no acute effects on *Daphnia magna* for concentrations 1 to 1000 mg L^{-1}.

Regarding the toxicity of target antimicrobials on aquatic plants, studies have been conducted with ciprofloxacin showing high toxicity to *L. minor* (the lowest EC_{50} calculated is 107 μg L^{-1}) (Robinson et al., 2005; Ebert et al., 2011), while Kołodziejska et al. (2013) reported no toxicity in a recent study for metronidazole for concentration up to 25000 μg L^{-1}.

Fish is the least studied aquatic organism category since the cost and timing of the experiments are relatively high compared to those of other organisms. Regarding the target antimicrobials, data is available only for trimethoprim, sulfamethoxazole and ciprofloxacin. According to the results, no toxic effects were reported for *Oryzias latipes* from trimethoprim and sulfamethoxazole (Kim et al., 2007; Park and Choi, 2008); as for *Gambusia holbrooki*no acute effects reported for concentrations up to 60 mg L^{-1} of ciprofloxacin (Martins et al., 2012).

1.1.4 Risk assessment from the occurrence of antimicrobials in the aquatic environment

The probability of occurrence of substance disturbances in human or in the environment when it comes in contact with it is determined as EnvironmentalRiskAssessment (ERA).

ERA contains the interactions of hazards, humans and ecological resources. It gives information for the risks for humans and ecosystems so ERA consists of two components; the risk assessment for human health and the ecological risk assessment (EMEA, 2006; European Commission, 2003). In the current PhD Thesis the investigated compounds are the antimicrobials and if they are not completely removed through the municipal wastewater treatment, then they are channeled directly into the environment with the potential to cause adverse effects on aquatic organisms. The results of such studies usually demonstrate adverse disturbances in non-target organisms, especially when present at higher concentrations than expected concentrations in the environment. There are not enough eco-toxicological studies on antimicrobials compounds, and this is a major gap in knowledge of their effects, especially in the aquatic environment. For that reason, there are several ecotoxicological models which are widely applied for the determination of ERA (e.g.ECOSAR, EPI Suite).

The risk analysis is often determined by a Risk Quotient (RQ) value wherein, at the first step, the predicted environmental concentration (PEC) and the concentration that causes no effect on specific organisms (PNEC) are calculated. Afterwards, the division of PEC to PNEC allows the determination of RQ value for each studied compound. When the RQ value is greater than the unit, then an environmental threat is possible for the aquatic environment (EC, 2003). For mixtures of micropollutants, the sum of the RQs of the studied substances gives the total toxicity they cause to the aquatic environment(Hu et al., 2021d; Ferrarietal., 2004; Carlssonetal., 2006). The estimation of environmental risk assessment in aquatic organisms from the most used antimicrobials in Greece is one of the objects of the current PhD Thesis.

1.1.5 Occurrence of antimicrobials in wastewater

The first report on the presence of antimicrobialsin the environment was published in England in 1982 and reported the detection of macrolides, tetracyclines and sulfonamides in river water samples (Sarmah et al., 2006). Since then, there are many reports on the presence of antimicrobials in various aqueous samples, such as surface water, groundwater, seawater, drinking water, treated wastewater, and hospital wastewater (Souza et al., 2017; Homem and Santos, 2011). Typically, antimicrobial substances are detected at concentrations ranging from several hundred ng L^{-1} to a few tens of μg L^{-1} in the aquatic environment. Trace amounts

of antimicrobials have been detected in surface water of US and Europe during the last threedecades. As expected, the highest concentrations have been found in hospital wastewater (Halling-Sorensen et al., 2000; Gros et al., 2010; Kümmerer, 2009c; Le-Minh et al., 2010; Oulton et al., 2010; Escher et al., 2011; Homem and Santos, 2011).

Many studies for the occurrence of antimicrobials have been conducted in Europe, North America, East Asia and Australia. According to the studies of the last decade, six major classes of antimicrobials (β-lactams, quinolones, macrolides, tetracyclines, sulfonamides and other antibiotics) have been detected at the influents and effluents of urban wastewater treatment plants (Hu et al., 2021;Watkinson et al., 2007; Kümmerer, 2009c; Zhang and Li, 2011).

Among the target compounds SMX, as well as its metabolite N-acetylsulfamethoxazole, are the most often detected antimicrobials. They have been found in several wastewater treatment plants worldwide, while the highest concentrations have been detected in Switzerland (1100 ng L^{-1}) and Great Britain (2200 ng L^{-1}). According to several studies, the metabolite of SMX is considered as more dangerous than the parent compound (Göbel et al., 2005; Li and Zhang, 2010). The presence of trimethoprim in wastewater varies between 3000 ng L^{-1} and 8000 ng L^{-1}, this compound has been detected in different European countries as well as in USA, China, Australia and Hong Kong (Le-Minh et al., 2010 Zhang and Li, 2011). Ciprofloxacin has been detected at low concentrations but also in high frequency. CIP is one of the most frequently detectable chemicals in the effluents of wastewater treatment plants Europe (Loos et al., 2013) andinAustralia (Watkinson et al., 2007). The highest concentrations were determined in Australia (4600 ng L^{-1}) and in Hong Kong (7870 ngL^{-1}) (Watkinson et al., 2007; Xiao et al., 2008; Li and Zhang, 2010). In European STPs the concentrations of SMX, TRI and CIP range between 91 to 794 ng L^{-1}, 99 to1264 ng L^{-1} and 40 to 3353 ng L^{-1}, respectively (Gavrilescuet al., 2015).

1.1.6 Fate and removal of antimicrobials in STPs systems

The removal of antimicrobial substances during wastewater treatment has been investigated for several substances of this class of pharmaceuticals. According to the existing knowledge, the majority of antimicrobials are partially removed during the secondary treatment of sewage, while most of them are removed significantly during tertiary treatment with ozone or

activated carbon. However, it should be noted that their behavior during conventional wastewater treatment is not fully known since the role of adsorption and biodegradation mechanisms has not been studied extensively. In addition, there are many gaps in the literature for the parameters that affect their removal and the production of transformation by-products. Antimicrobials'removal capacity seem to be affected by the ambient temperature. That's because in colder countries, their frequency detection and their concentrations levels are higher comparing to the hottest countries. According to the literature, temperature is a parameter that contributes to the different removal rates observed for different substances (Watkinson et al., 2007; Le-Minh et al., 2010; Oulton et al., 2010; Homem and Santos, 2011).

Beta-lactams, are considered as the most unstable antimicrobial substances as they may be hydrolysed. So far, it is not known whether they produce transformation products during biological treatment and whether these compounds are toxic (Watkinson et al., 2007; Le-Minh et al., 2010; Zhang and Li, 2011). The removal capacity of sulfonamides by conventional wastewater treatment plants is lower than 25% (Le-Minh et al., 2010; Li and Zhang, 2010). In most of the studies, it has been reported that sulfamethoxazoleis eliminated at a very low rate of 20% during biological treatment, although there are some studies reporting removal efficiencies between 55 and 74% (Le-Minh et al., 2010; Li and Zhang, 2010). Its metabolite, N_4-acetylsulfamethoxazole, has been detected at the effluent wastewater as well as at the inlet of conventional wastewater treatment plants. For that reason, it is speculated that this compound can be produced during both human metabolism and biological wastewater treatment. (Göbel et al., 2005; Watkinson et al., 2007; Le-Minh et al., 2010; Li and Zhang, 2010). Regarding, trimethoprim, Zhang and Li (2011) estimated that it was removed by adsorption in an activated sludge system at a percentage between 19% and 26%, while no biodegradation was observed. According to many studies, the removal of quinolones occurs mainly through the mechanism of adsorption rather than biodegradation (Kümmerer et al., 2000; Le-Minh et al., 2010; Zhang and Li, 2011). Lindberg et al. (2006) estimated removal of ciprofloxacin equal to 44% in activated sludge systems due to adsorption. An other study indicates that ciprofloxacin removed at 85% through adsorption, while no removal was observed through the biodegradation mechanism (Zhang and Li, 2011). Halling-Sorensen et al. (2000) reported that some antibiotic substances such as penicillins and ampicillins can be biodegraded easily but other substances such as erythromycin, metronidazole and sulfamethoxazole may not be destroyed readily by conventional

wastewater treatment. As for cefadroxil, there is no mention of its behavior and whether it is removed by conventional wastewater treatment methods.

1.2 Duckweed: *Lemna minor*

1.2.1 Uses and properties of *L. minor*

Duckweeds are the smallest and fastest growing plants. They are monocotyledons belonging to the family *Lemnaceae*. Duckweeds are classified as higher plantsor macrophytes, in some cases are often mistaken for algae. The word *Lemnaceae* derived from the Greek word 'Limne' which meaning is pond. Duckweeds prefer standing water or water moving with slow velocity and form dense groups. They grow more in spring and autumn, while in summer, due to the heat, their growth is slower. The range of water temperatures that can grow is between 6 to 33 °C (OECD, 2006; Landolt, 1986).

They are commonly found in areas with nutrient-rich waters, even in stagnant waters that are dried during the summer. When the water becomes too little, then for a while, they can be preserved with their roots in the mud. What is not easy to tolerate is the ice, but in the Mediterranean climate these climate conditions are not so common. Compared with older plants, younger duckweeds tend to be paler, colored light green, have shorter roots and consist of 2 to 3 leaves of different size whose diameter ranges from 1.5 to 5 mm. Duckweed consists of four genera: *Lemna*, *Spirodela*, *Wolffia* and *Wolffiella*. *Lemna minor (L. minor)* belongs to Class: Liliopsida (Submission: Arecidae) in the Family of *Lemnaceae* and is the largest genera of the family. As for its structure, it has a discoid stem and a very thin root which emanates from the center of the lower surface to each foliage. Rarely these species produce flowers and the plants reproduce stem new foliage (OECD, 2006; Landolt, 1986; Fairchild et al, 1997).

Specifically, *L. minor* is a natural filter of water, as it absorbs nutrients from the water which are responsible for algae growth. It is generally considered that aquatic plants have great prospects to function as bio-reservoirs and biofilters in the field of water pollution due to their abundance and limited mobility. They have been successfully used to selectively isolate heavy metals and nutrients through their root system and by suction into their body (Olette et al, 2008). *L. minor* is probably the most commonly used aqueous vascular plant organism for toxicological experiments because of the following advantages; ease of cultivation, small

size, fast growth, simple structure, ease of collection and rapidly export of the results (Fairchild et al, 1997, OECD, 2006; Kiss et al, 2001).

*L. minor*is a fast-growing plant and is found from the tropics to the tropical zone. As a primary producer, it is a source of feed for birds, fish and small animals. Additionally, as a regulator of the oxygen level, it plays a significant role to several small invertebrates but also to the biogeochemical cycles of elements in aquatic ecosystems. Because of its rapid growth, it covers a large surface with its foliage and this prevents organisms under the surface of the water to photosynthesize. This causes a competitive factor against the other aquatic plants that need solar radiation (Horvat et al, 2007).

1.2.2 Use of *L. minor* as an indicator of toxicity

Numerous toxicological studies have been carried out over the last few decades using several aquatic organisms among them and duckweeds. *L. minor* is extensively used as a test organism for finding the toxicity of various "dangerous - toxic" substances. Compared to other aquatic organisms, such as *E. Canadensis* and *C. aquatica*, the *Lemnaceae* species present greater precision in the results and better reproducibility (Olette et al, 2008).

Toxicological studies conducted using *L. minor* are mainly done in indoor aquatic microcosms under controlled and regulated conditions (Gorzerino et al, 2008). So far, most of the studies that have been carried out investigate the effects ofpesticides and heavy metals on target organism as individual substances (Wenhua et al, 2006). *L.minor* has been extensively used for studying the ecotoxicity of herbicides as it is considered a non-target organism for these compounds (Bottcher et al, 2006). Furthermore, it has been observed that at very low concentrations of heavy metals (copper, cadmium, iron) there is no deleterious effect on the mechanism of *L. minor* (Wenhua et al, 2006).

1.2.3 Use of *L. minor* in constructed wetlands

According to the definition for wetlands that given in the Ramsar Convection: *"Wetlands include a wide variety of habitats such as marshes, peat lands, floodplains, rivers and lakes, and coastal areas such as salt marshes, mangroves, and sea grass beds, but also coral reefs and other marine areas no deeper than six metres at low tide, as well as human-made wetlands such as waste-water treatment ponds and reservoirs."*. Wetlands have a number of

roles in the environment, mainly water purification, flood control, carbon sink and shoreline stability. Wetlands arethe link between land and water presenting the greatest biologically diversity between all ecosystems (Ramsar Convention on Wetlands, 2013).

Mostly, in developing countries the treatment of domestic wastewater remains a substantial problem. Constructed wetland technology can be used to treat municipal and industrial wastewater; itis a promising alternative treatment process that is widely used to remove conventional and non-conventional pollutants such as nutrients and metals from wastewater. Furthermore, constructed wetland technology has been widely used for tertiary wastewater treatment (Nuamahet al., 2020; Matamoros et al., 2012; Ran et al., 2004).

Duckweed-based wastewater systems are inexpensive to install and to operate. Furthermore, as it was mentioned above, *L. minor* have longer and faster growing periods compared to most of the other aquatic plants. Previous studies have shown that among many plant-based systems the duckweeds contribute into highly removal efficiencies of heavy metals and some organic microcontaminants through biodegradation, plant uptake and photodegradation. Especially, duckweeds are capable to treat swine effluents due to their tolerance to high nutrient levels (Reinhold et al. 2010; Haarstad et al. 2012; Zhang et al. 2014; Sekomo et al. 2012; Oporto et al., 2006; Matamoros et al., 2012; Ran et al., 2004).

Furthermore, duckweed-based wastewater treatment seems to be an effective alternative method compared with conventional treatment systems that treats domestic and industrial wastes. The last decade, phytoremediation seems to be one of the successful methods that lead to green sustainability. Also, duckweed ponds revealed a potential for polishing and valorization of domestic wastewater (Shirinpur-Valadiet al. 2019; Haarstad et al. 2012; Zhang et al. 2014; Sekomo et al. 2012; Ran et al 2004).

Constructed wetlands with duckweed (*L. minor*) have been applied with success in different countries for the removal of nutrients and organic matter (Reinhold et al. 2010; Haarstad et al. 2012). The use of *L. minor* treatment systems is growing during the last years, however, there is still limited knowledge on their efficiency to remove organic micropollutants as well as on the mechanisms affecting their removal (Reinhold et al. 2010; Matamoros et al., 2012). As for antimicrobials, so far, to the best of our knowledge there is no information for the capacity of duckweed systems to remove these compounds.

1.2.4 Reuse and exploitation of *L. minor*

Energy problems in developed and developing countries demand the adoption of renewable, cost-effective and eco-friendly technologies. Under this frame, several plants have been used for bioenergy production. One of the most commonly used plant for this purpose is wheat which has been used for many years for bioethanol production. However, the cost for production of bioethanol is still too high (Abbasi and Abbasi, 2010; Littlewood et al., 2013; Talebnia et al., 2010; Miranda et al., 2016). As a result, there is need for new processes, which can efficiently utilize new seeds/plants for bioethanol production with lower costs.

L. minor can produce very high amounts of biomass, especially when the cultivation is on nutrient rich wastewater such as municipal or swine wastewater. Recent studies have studied duckweed for its energy efficient and simultaneous use in wastewater treatment systems. *L. minor* has been used as animals' feedstock and also for biofuel sproduction. Duckweeds can produce biomass with high content in crude protein due to their ability of direct metabolism of ammonia from water body. Domestic wastewater contains nitrogen and phosphorous originating from human urinethat can be used for nutrients recovery and crop production (Saliu et al., 2021; Liu et al., 2013, Zhang et al., 2013). Additionally, they can accumulate high percentage of its dry weight in starch which makes possible the production of bioethanol from biomass. According to previous studies, the starch content in duckweed ponds containing agricultural orswine wastewater was in a range of 12.5–52.9% (Liu et al., 2018; Wang et al., 2014; Ge et al., 2012; Mohedano et al., 2012; Xiao et al., 2013; Xu et al., 2011; Xu and Shen, 2011).

So far, there are only a few studies that use *L. minor* for simultaneous nutrient removal and valorization of the produced biomass in tertiary wastewater systems. Additionally, to the best of our knowledge, there is no information for the use of human urine for *L. minor* cultivation.

1.3 Aims and outline of PhD Thesis

The main objectives of the PhD Thesis were (a) to estimate the potential environmental risks associated with human antimicrobials consumption in Greece, (b) to investigate the removal of nutrients and selected antimicrobials in urine and treated wastewater cultivating with *L. minor*, (c) to estimate biomass production with simultaneous crude protein and starch production in continuous flow reactors planted with two duckweeds and (d) to investigate the fate and removal of antimicrobials compounds in planted reactors with *L. minor* through

batch and continuous flow experiments. Four scientific works were conducted to achieve the goals of the current study and their results were published in the following Papers I to IV. Specifically:

(a) Consumption data of Greece was collected for the 24 most often used antibiotics for three years 2008-2010 and their Predicted Environmental Concentrations (PECs) in raw and treated wastewater were calculated. The ecotoxicological risk (RQ) was estimated by calculating the ratio of PEC to Predicted No Effect Concentration (PNEC) for three aquatic organisms (algae, daphnids and fish) (**Paper I**).

(b) Experiments with duckweed *L. minor* were conducted using treated wastewater and different types of urine (fresh, hydrolyzed, stored, and synthetic). The effect of several parameters such as urine dilution, temperature, existence of macro- and microelements on growth rate was investigated. The efficiency of *L. minor* to remove nutrients (COD, total N, NH_4^+-N, total P) and selected antimicrobials (sulfamethoxazole, SMX and ciprofloxacin, CIP) from human urine and treated wastewater was studied. Furthermore, the content of produced biomass on protein and starch was determined (**Paper II**).

(c) The growth rate of two species of duckweeds (*L. minor*, *L. gibba* and the combination of both species) were estimated by the cultivation of themin secondary treated wastewater without and with the addition of 30 mg L^{-1} NH_4-N. The purpose was to achieve the following goals: rapid growth of duckweeds for biomass production, removal of nutrients (COD, TN, TP, NH_4-N) and effective increasement of crude protein and starch in tested duckweeds for possible future use in feed / biofuels (**Paper III**).

(d) The removal of four antimicrobials, cefadroxil (CFD), metronidazole (METRO), trimethoprim (TMP) and sulfamethoxazole (SMX) from treated wastewater using *L. minor* bioreactors were investigated. The main scope was to identify plant and not plant-associated processes responsible for their elimination from secondary treated wastewater. After investigating the potential toxicity of antimicrobials in the organism, batch and continuous flow experiments were carried out. Through batch experiments the role of photodegradation, hydrolysis, sorption and plant uptake on target

compounds removal was studied. Degradation kinetics of target compounds were calculated and the transformation by-products were identified. A continuous flow lab-scale system planted with fresh *L. minor* was used to investigate the removal of two target compounds (METRO and TMP) from secondary treated wastewater in different ponds (**Paper IV**).

2 Experimental and Analytical methods

All the experimental procedures that will be described in the following paragraphs of Chapter 2 were conducted in the Water and Air Quality Laboratory and the Cultivation Room of the Department of Environment, University of the Aegean.

2.1 Experimental procedures

2.1.1 Antibiotic consumption in Greece and Environmental Risk Assessment (**Paper I**)

In the current study, 24 representative substances were selected by the categories of antimicrobials which are the most used in Greece. For that reason, home medication sales' data from IMS Health Incorporation were collected for years 2008 - 2010 and the average annual number of sold medicine boxes containing injectable, oral (capsules, tablets and suspensions) and other forms of target drugs (e.g. crèmes/gels/ eye drops) was calculated.

The amounts of these substances that are excreted unchanged in urine were calculated using excretion rates from the literature. The total sum of each active ingredient was multiplied with the corresponding excretion rate in order to calculate the excreted production (kg) which end ups in the raw wastewater of conventional wastewater treatment plants. To calculate the concentrations of antimicrobials in treated wastewater, the removal rates of these compounds during wastewater treatment were collected after literature review. For the antimicrobial substances that removal efficiencies' data was not available in the literature, the values were calculated using EPI Suite Interface program version 4 (based to EPA methods). For calculating the amounts of antimicrobials that are released to the sewerage system, it was assumed that all the amounts of sold antimicrobials in Greece are consumed by the patients.

According to the Technical Guidance Document of the European Commission on Risk Assessment (2003), the Predicted Environmental Concentration (PEC, as Kg m^{-3}) was estimated for antimicrobials in raw wastewater, treated wastewater and river water. For estimating dilution factors (D) in Greek rivers, flow rates data were collected from the Greek STPs discharging treated wastewater in rivers. In the current study, the PEC of antimicrobials was calculated for three cases: raw sewage, treated wastewater and aquatic environment (river water), applying the corresponding conditions in each case. The daily flow rate of wastewater per capita was considered equal to 0.2 m^3.

To calculate the Predicted No-Effect Concentration (PNEC) of the target compounds, a literature review was initially conducted for acute toxicity data of the 24 investigated antimicrobials to fish, daphnia, and algae. For the substances that there was no data available in the literature, toxicity data was estimated using SARs (Structure Activity Relationships) predicted model ECOSAR (ECOWIN v.1.00). PNEC was calculated by dividing the chosen acute endpoint value by a suitable assessment factor (AF).

The risk quotient (RQ) is an indicator of the ecotoxicological risk and is a ratio between PEC and PNEC for each substance. In cases that RQ is greater than 1, ecotoxicological risk for the aquatic environment is expected. On the other hand, values of RQ less than 1 indicate no ecotoxicological risk for the aquatic environment and further research is not needed (Technical Guidance Document of the European Commission, 2003).

Further information for the calculations of Environmental Risk Assessment in Greece is available in **section 3.1**.

2.1.2 Cultivation of *Lemna minor* in urine and treated wastewater (**Paper II**)

Thecultivation of *L. minor* in human / synthetic urine and treated wastewater was studied. For this reason, the effect of several parameters (urine dilution rate, temperature, addition of microelements) on duckweed growth rate was investigated. The ability of *L. minor* to remove nutrients (NH_4-N, P) and two selected antimicrobials (sulfamethoxazole and ciprofloxacin) from all tested media wasalso studied.

Duckweed *L. minor* was grown on diluted human urine (fresh, hydrolysed and stored for 1 day), synthetic urine and treated wastewater. Batch experiments were initially done using different dilution factors (1:2, 1:5, 1:10, 1:25, 1:50, 1:100, 1:150, 1:200, 1:250) of human and synthetic urine in order to calculate the growth rate of *L. minor* under different conditions. Experiments were also done at different temperatures (12^{o}C, 18^{o}C and 24 ^{o}C), different initial mass of duckweed and addition of different concentrations of trace elements such as Cu, Fe, Mn, Mg etc. Afterwards, the best urine solution was selected and the crude protein and starch content of biomass were determined. Specifically, four different media were used to investigate fresh biomass production, starch and crude protein content, removal of nutrients and aqueous removal of the antimicrobials. The four tested media were: the Swedish

Standard medium (SIS), 1:200 Human urine (HU), 1:200 Human urine (HU harv.) (harvesting of biomass every 5days during the experiment) and secondary treated wastewater (ww).

The elimination of urea, NH_4^+, COD and TP in all tested media, the removal of selected antimicrobials and the characterization of the produced biomass in crude protein / starch content were determined.

Further information for the experiments with *L.minor* cultivated in urine and treated wastewater is availablein **section 3.2**.

2.1.3 Cultivation of *Lemna minor* and *Lemna gibba* in treated wastewater (**Paper III**)

Experiments were performed using secondary treated wastewater and two photosynthetic organisms belonging to duckweeds (*L.minor* and *L.gibba*). Three continuous flow / batch experiments were performed (lasting 53 days) wherein each one divided into three phases (A, B and C). In the first Experiment *L.minor* was used, *L.gibba* was used in the second while the combination of those two duckweeds was used in the third. During Phase A of each experiment, treated wastewater were used, while during Phase B 30 mg L^{-1} NH_4-N were added in treated wastewater to enhance biomass production (Wang et al.,2014).

During the experimental phases A and B, daily and weekly measurements were performed. In the continuous flow system, pH (input, tank, output), T (room and tank temperature) and the flow rate of the system were measured in a daily basis.In aweekly basis, the total biomass was calculated and the excess biomass was removed in order to maintain the initial volume of the biomass (13 gr). Aqueous samples for the determination of COD, NH_4-N, TN and TP and biomass samples were also taken weekly for investigating the performance of the system and determining the crude protein and starch content of the biomass, respectively.

At Phase C, starch accumulation experiments were conducted using 100 mL tap water in petri dishes and initial mass of duckweeds equal to 2 g (transferred from each of three tanks, respectively). Each duckweed individually and their combination were tested in triplicates. The total duration of those experiments was 21 days and the starch content was determined at Days 0, 7, 14 and 21.

Further information for the experiments with the two duckweeds, crude protein and starch content is available in **section 3.3**.

2.1.4 Batch and Continuous flow experiments using reactors planted with *Lemna minor* (**Paper IV**)

Toxicity range finding tests were initially conducted to check the possible effects of the target compounds (cefadroxil, metronidazole, trimethoprim and sulfamethoxazole) on *L. minor* individually as well as in mixture (OECD, 2006).

Batch experiments

Afterwards, four different reactor systems were used to investigate the aqueous removal of target antimicrobials and to clarify the role of biotic and abiotic mechanisms on their removal (hydrolysis, photodegradation, sorption and plant uptake). The flasks were placed in incubator chambers under constant light for a period of 24 days. The temperature was set at 24 ± 0.5˚C, pH was 7.0 ± 0.2 and the initial concentration of the compounds was equal to 250 μg L^{-1}. For the investigation of antimicrobials uptake by *L. minor*, 2 gr of fresh organism were added in each flask. In order to investigate the sorption of target compounds in test organism, *L. minor* communities were exposed to 1 g L^{-1} sodium azide for 7 days prior the addition to experimental reactors (Reinhold et al. 2010). Experiments were also conducted in the absence of *L. minor* under light and dark conditions to estimate the role of photodegradation and hydrolysis on the removal of antimicrobials, respectively.

Experiments for identification of by-products

Following the same procedure as in batch experiments, two different batch reactors were used for all target compounds individually to investigate their transformation by-products in the presence or absence of *L. minor*. The flasks with no *L. minor* contained only the Medium SIS with the target antimicrobial compound, while the flasks with the duckweed contained the Medium SIS, the target substance and 2gr of fresh biomass.

Continuous flow experiments

Three duckweed lab-scale ponds were used in series under 16/8h light/darkness, respectively. The volume of each pond was 5 L, the hydraulic residence time was equal to 6.5 d, while *L. minor* biomass was added at a density of 600 g fresh weight per m^2 (Sekomo et al.

2012).Evapotranspiration losses were counterbalanced daily by adding tap water. After an initial start-up period of 3 months to stabilize the flow rate and to allow duckweed acclimatization and growth onto wastewater, wastewater was spiked with target antimicrobials in order to achieve a concentration of around 10 μg L^{-1} at the inlet of the lab-scale system. The system was operated under these conditions for a period of 79 days. The elimination of conventional pollutants as well as of target antimicrobials (metronidazole and trimethoprim) in each pond was investigated.

Further information for the experiments with *L.minor*, antimicrobial compounds and their by-products is available in **section 3.4**.

2.2 Analytical methods

The analysis of parent antimicrobial compounds and the chemical analysis of all tested parameters were conducted in Water and Air Quality Laboratory, Analytical Chemistry Laboratory and the Biology Laboratory of the Department of Environment, University of the Aegean. The analysis of the parent compounds and the by-products formed in continuous flow experiments described in Paragraph 2.1.4 was conducted in the Laboratory of Analytical Chemistry of the Department of Chemistry, National and Kapodistrian University of Athens.

2.2.1 Chemicals and Reagents

Analytical standards of CFD (cefadroxil), METRO (metronidazole), TMP (trimethoprim), SMX (sulfamethoxazole) and CIP (ciprofloxacin hydrochloride) were purchased from Sigma – Aldrich Fluka (Steinheim, Germany). Stock solutions were prepared in pure water (batch experiments) and in methanol, MeOH (continuous-flow experiments urine experiments).

Culture of *Lemna minor L.,* clone St. was donated by Federal Environment Agency (Berlin, Germany). All salts used for *L. minor* growth medium were purchased by Fluka (Heidelberg, Germany). The culture of *Lemna gibba L.* was collected from the island of Lesvos, within Natura area (GR4110012, North Lesvos), in a natural wetland at an altitude of about 400

meters. *L. gibba* was acclimatized in total for 6 weeks in tanks with secondary wastewater before the elaboration of the experiments that described in section 2.1.3.

HPLC grade water was prepared in the laboratory using a MilliQ/MilliRO Millipore system (Bedford, USA), while MeOH (LC-MS grade) was obtained from Fisher (USA). Strata – X polymeric reversed phase SPE cartridges (200mg/6ml) and Regenerated Cellulose (RC) filters (0.2 μm, 4 mm) for antimicrobials analysis were purchased from Phenomenex (Torrance, CA, USA). HU and secondary treated wastewater used in this study were collected from the University Campus (University Hill, Mytilene, Lesvos island, Greece).

Further information for duckweeds cultures and the reagents that were used is available in **sections 3.2.2.1, 3.3.2** and **3.4.2.1**.

2.2.2 Analysis of antimicrobials

The target compounds in urine experiments (2.1.2) and batch experiments (2.1.4) were analyzed by a Shimatzu (Japan) LC20-AD prominence liquid chromatographer associated with a SPD-M20A prominence diode array detector and a SIL-20AC auto sampler. The analytical procedure for all five antimicrobials was based on a previously published method (Ašperger et al., 2009).

For the determination of the target compounds in wastewater samples originating from continuous-flow experiments, solid phase extraction (SPE) was used according to Dasenaki and Thomaidis (2015) and they were analyzed through a liquid chromatography–tandem mass spectrometry (LC-MS/MS) system.

Further information for the analysis of antimicrobial compounds is available in **sections 3.2.2.3, 3.3.2** and **3.4.2.6**.

2.2.3 Analysis of antimicrobials by-products

An ultrahigh-performance liquid chromatography (UHPLC) system (DionexUltiMate 3000 RSLC, Thermo Fisher Scientific, Germany) coupled to a quadrupole-time-of-flight mass

spectrometer (QTOF-MS) (Maxis Impact, BrukerDaltonics, Bremen, Germany) was used for the screening analysis and the identification of candidate transformation products (TPs) of selected antimicrobial compounds.

Further information for the procedure that was followed is available in **section 3.4.2.6** and in supplementary materials in **section 6.4.1**.

2.2.4 Analysis of other parameters

The determination of chemical oxygen demand (COD), biological oxygen demand (BOD), ammonia-N (NH_4-N), nitrate-N (NO_3-N), total phosphorous (TP), total nitrogen (TN) and total suspended solids (TSS) in aqueous samples was conducted according to Standard Methods (APHA-AWWA-WPCF, 2005). Temperature (T), dissolved oxygen (DO), conductivity and pH were measured using portable instruments. The urea determination was based to the modified diacetylmonoxime colorimetric assay (Mulvenna and Savidge, 1992; Rozet et al., 2007), while an Ion Chromatography system with suppressed conductivity detection was used for the determination of NH_4^+-N and other cations (Na, K, Mg, Ca) for the characterization of HU composition. Starch content in duckweed samples was determined according to anthrone method (Hansen and Møller, 1975), while calculation of crude protein was based on the measurement of TN concentration in biomass (Xiao et al., 2013).

Further information for the analysis of all parameters that were determined in current PhD dissertation is available in **sections 3.2.2.3, 3.3.2 and 3.4.2.6**.

2.2.5 Calculations and data analysis

For data evaluation, OriginPro 8 SR0 (Version 8.0724, OriginLab Corporation, Northampton, USA) was used wherein all graphs were constructed. The results were statistically checked with SPSS 17.0 by one-way ANOVA and paired-samples T-test. When ANOVA was significant at $p<0.05$, the Tukey's HSD post hoc test was run to identify differences between the tested parameters. Furthermore, several equations were used for the treatment of experimental results and calculation of different constants.

Further information for the appropriate statistical analysis, the protocols that followed and the equations that were used in the current PhD dissertation is available in **sections 3.1.2, 3.2.2.4, 3.3.2 and 3.4.2.6**.

3 Results and Discussion

3.1 Environmental Risk in Greece due to antimicrobials in wastewater (Paper I)

3.1.1 Introduction

Antimicrobials are a significant group of pharmaceuticals that are extensively used by humans and animals against microbial infections. After their consumption, human use antimicrobials are metabolized to an extent ranging from 10% to 90% and they are excreted through urine and faeces into sewage either as unchanged parent molecules or as metabolites. Due to their partial elimination during wastewater treatment, trace amounts of these compounds (ng L^{-1} to μg L^{-1}) have been detected in treated wastewater and surface water worldwide (Zhang and Li 2011; Michael et al. 2013; Thanh Thuy and Nguyen 2013), and they represent a possible threat for the aquatic environment due to their acute and long term toxicity (Halling-Sørensen et al. 1998; González-Pleiter et al. 2013).

Considering the great number of antimicrobials that are commonly used globally and the high cost for the implementation of extensive national monitoring programs, the analytical determination of their concentrations is a challenging matter. For this reason, consumption data is often used to estimate concentrations of antimicrobials and other pharmaceuticals in wastewater and aquatic environments (Stuer-Lauridsen et al. 2000; Jones et al. 2002; Carlsson et al. 2006; Lee et al. 2008; Turkdogan and Yetilmezsoy 2009; Besse et al. 2012; Ortiz de Garcia et al. 2013). In such studies, the reliability of estimated concentrations is affected by the reliability of pharmaceuticals' consumption data as well as by the assumptions adopted concerning their excretion rates by humans and their removal efficiencies in Sewage Treatment Plants (STPs).

In Greece, published data for the occurrence of antimicrobials in wastewater and the aquatic environment is very limited. Botitsi et al. (2007) reported the existence of some sulfonamides and trimethoprim in treated wastewater samples originating from four Greek STPs. Moreover, in an other study, Kosma et al. (2014) determined elevated concentrations of sulfamethoxazole and trimethoprim in eight STPs located in different Greek cities and estimated a high acute risk for fish, invertebrates and algae. So far, there is no information for the consumption and expected concentrations of other antimicrobials in Greece. As a consequence, the environmental risk due to the disposal of domestic wastewater containing antimicrobials in Greek aquatic environments has not been estimated to date.

Based on the above, the main objectives of this study were to predict the concentrations of antimicrobials in Greek wastewater and to estimate the potential environmental risks associated with consumption of antimicrobials by humans. For this reason, consumption data was collected for three consecutive years for the 24 most used antimicrobials in Greece. Based on their excretion rates and municipal wastewater production, the expected concentrations of antimicrobials in raw wastewater were calculated. It should be mentioned that as no excretion data was available in the published literature for all different formulations of each drug, a typical excretion rate was used for each compound. Keeping in mind the removal efficiency of these compounds in conventional STPs, the expected concentrations of antimicrobials in treated wastewater were also calculated. Acute and chronic toxicity data was collected from peer-reviewed literature or calculated using the predictive ECOSAR model, and the potential environmental risk due to the disposal of raw and treated wastewater to the aquatic environment of Greece was estimated. Using ECOSAR, the potential risk resulting from the exposure to the mixture of all target antimicrobials was also estimated.

3.1.2 Materials and Methods

3.1.2.1 Consumption of antimicrobials in Greece

In the current study, the 24 most often used antimicrobial compounds in Greece were studied (Table 3.1.1). For this reason, home medication sales' data from IMS Health Incorporation were collected for years 2008, 2009 and 2010 and the average annual number of sold medicine boxes containing injectable, oral (capsules, tablets and suspensions) and other forms of target drugs (e.g. crèmes/gels/ eye drops) was calculated. The average annual consumed amount of each antimicrobial compound was calculated according to Equation 3.1.1.

$$Consumed\ Amount\ (Kg/year) = \sum \frac{Dosage}{Form\ of\ drug} \times \frac{Number\ of\ drugs}{Medicine\ box} \times N \qquad (3.1.1)$$

Where:

Dosage/Form of drug: the mass of target antimicrobial contained in each capsule/tablet/suspension/injectable (Kg)

Number of drugs/Medicine box: the number of capsule/tablet/injectable contained in each box

N: Number of medicine boxes sold per year

3.1.2.2 Concentration of antimicrobials in wastewater and river water

For calculating the amounts of antimicrobials that are released to sewerage system, it was assumed that all amounts of sold antimicrobials in Greece are consumed by the patients. Based on this assumption, it was neglected the amounts of antimicrobials that are disposed unused in municipal solid waste or to sewerage system. The amounts of antimicrobials excreted unchanged in urine were calculated using excretion rates reported in the literature (Table S3.1.1). In cases that published data on excretion rates of target compounds was not consistent, the highest suggested excretion rates were taken. Only parent compounds were regarded and no calculations were made for the produced metabolites, since information on excreted metabolite fractions is highly variable (Escher et al. 2011). The annual excreted amounts per antimicrobial were calculated by multiplying the consumed amounts of target compound (Kg $year^{-1}$) with the relevant excretion rate (ER) by using the following equation (Equation 3.1.2):

$$Excreted\ Amount\ (Kg/year) = \text{Consumed Amount} \times \text{ER}\ (\%) \quad (3.1.2)$$

According to the Technical Guidance Document (TGD) of the European Commission on Risk Assessment (2003), the Predicted Environmental Concentration (PEC, as Kg m^{-3}) in wastewater and water was calculated from the following equation (Equation 3.1.3; European Commission 2003):

$$PEC = \frac{A \times \left[1 - \frac{R}{100}\right]}{365 \times P \times V \times D} \quad (3.1.3)$$

Where,

A: the predicted amount of target compound that is excreted per year in sewage of relevant geographic area (Kg $year^{-1}$)
R: the removal rate (%) of target compound during conventional biological wastewater treatment
P: the total number of inhabitants (permanent and tourists) for the geographic area considered (inh)
V: the produced volume of wastewater per inhabitant and day ($m^3\,inh^{-1}\,day^{-1}$) and
D: the dilution factor of wastewater by surface water flow

The removal rates (R) of the target compounds during conventional wastewater treatment were identified from the literature (Table S3.1.1). For antimicrobials for which no relevant information was available, the European Protection Agency (EPA) method for total removal efficiency of EPI Suite Interface program version 4 was used (US EPA). Population data of Greece (permanent inhabitants, number of tourists) was taken from the Hellenic Statistical Authority (2011); whereas the produced volume of wastewater per permanent inhabitant/tourist and day was considered equal to 0.2 m^3 (Grung et al. 2008; Turkdogan and Yetilmezsoy 2009).
To estimate the concentrations of antimicrobials in raw wastewater, treated wastewater and river water, Equation 3.1.3 was solved for the following three cases: $R = 0$ and $D = 1$ (raw sewage); $R \neq 0$ and $D = 1$ (treated wastewater) and $R \neq 0$ and $D > 1$ (river water). For estimating dilution factors (D) in Greek rivers, data on flowrates was collected from the Greek STPs discharging treated wastewater in rivers.

3.1.2.3 Environmental risk assessment

Acute and chronic toxicity data was collected from the literature for the target compounds and for three different trophic levels (algae, daphnids and fish). For antimicrobials where more than one toxicity data was available, the lowest value was chosen in order to estimate the ecological threat for the worst-case scenario. For the compounds that no relevant information was available, toxicity data was estimated using ECOSAR (US EPA). In these cases, physicochemical properties of antimicrobials were initially evaluated from EPI Suite Interface Program and afterwards these values were introduced to ECOSAR model. The

physicochemical parameters used in ECOSAR are shown in Table S3.1.2; whereas acute and chronic toxicity data is presented in Tables S3.1.3 and S3.1.4, respectively.

The Predicted No-Effect Concentration (PNEC) for each compound and aquatic organism was estimated by dividing the chosen endpoint value (acute or chronic) by a suitable Assessment Factor (AF) (Equations 3.1.4a and 3.1.4b). As end points, the Effective Concentration 50% (EC_{50}), the Lethal Concentration 50% (LC_{50}), the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) were used. According to the Technical Guidance Document (TGD) on Risk Assessment (European Commission 2003), the AF for acute PNEC calculation was equal to 1000 for each of the three trophic levels. For chronic PNEC calculation, AF was equal to 100, 50 or 10 according to the number of species that toxicity data was available (European Commission 2003; Saling et al. 2005; Turkdogan and Yetilmezsoy 2009).

Acute PNEC calculation Chronic PNEC calculation

$$PNEC = \frac{EC_{50} / LC_{50}}{1000} \quad (3.1.4a) \qquad PNEC = \frac{NOEC / LOEC}{AF} \quad (3.1.4b)$$

After calculating PECs and PNECs values, Risk Quotients (RQs) were estimated for each antimicrobial and aquatic organism as indicators for ecotoxicological risk (Equation 3.1.5).

$$RQ = \frac{PEC}{PNEC} \quad (3.1.5)$$

In general, when RQ < 1 no ecotoxicological risk is expected, while for cases that RQ > 1, an ecotoxicological risk for the aquatic environment is considered possible (EMEA, 2006).

Additionally to the risk from individual antimicrobials, the risk from the mixture of all target compounds was also estimated. It is known that mixtures with components exhibiting the same mode of action act according to the model of concentration addition and this concept has been sufficiently used in the past for several mixtures of pharmaceuticals (Escher et al. 2011). According to Van Wezel and Opperhuizen (1995), all chemicals exert a baseline toxicity (narcosis) effect, independently from their specific mode of toxic action. There is typically a threshold concentration below which the specific mode of toxic action is not observed and effects are due to baseline toxicity. The exact mechanism of baseline toxicity is

not known; however it seems to be related to partitioning in membranes and adsorption to macromolecules. In mixtures, the contribution of the specific mode of action to the total toxicity decreases, while that for the baseline toxicity increases (ECETOC, 2001). Based on the above, to estimate mixture's effects, the baseline toxicity was calculated via ECOSAR for each antimicrobial. Then, RQ_{mix} for each aquatic organism was calculated using Equation 3.1.6:

$$RQmix = \sum_{i=1}^{n} RQi = \sum_{i=1}^{n} \frac{PECi}{PNECi} \quad (3.1.6)$$

3.1.3 Results and Discussion

3.1.3.1 Consumptions and emissions of antimicrobials to wastewater

The average annual sales of antimicrobials in Greece for years 2008-2010 was estimated to be 200,64,171 medicine boxes and the corresponding average consumed amounts were 119,026 Kg per year (Table 3.1.1, Figure S3.1.1). Higher sales were observed for b-lactams, cephalosporins, fluroquinolones and macrolides and specifically for amoxicillin, clarithromycin, cefuroxime axetil, ciprofloxacin and cefaclor. Similarly to the above, in a study in Turkey, higher consumption of antimicrobials was observed for cephalosporins and macrolides (Turkdogan and Yetilmezsoy 2009), while in Korea, cefaclor and amoxicillin were the most often consumed antimicrobials (Lee et al. 2008). Regarding the form of medication, 69% of sold antimicrobials in Greece were in the form of capsules and tablets; whereas 20% were used as suspensions (Figure S3.1.2a). The exceptions on this trend were netilmicin, erythromycin, ofloxacin, ceftriaxone, meropenem, clindamycin and amikacin that were mainly consumed as crèmes/gels (Figure S3.1.2b).

The estimated excreted amounts of target compounds in wastewater are presented in Table 3.1.1. According to the results, the highest excreted amounts were calculated for amoxicillin, clarithromycin, cefuroxime axetil, cefaclor, ciprofloxacin, cefprozil and metronidazole ranging from 3,480 to 22,631 Kg / year / compound. However, it should be acknowledged that high sales of an antimicrobial agent does not result necessarily to analogous high emissions to wastewater, as emissions depend on the content of target compounds in

medicinal boxes as well as on excretion rates from the human body. For instance, despite the high sales of clindamycin and azithromycin, their excreted amounts were estimated to be only 401 and 108 Kg per year, respectively (Table 3.1.1). These low emissions are due to the low excretion rates of these compounds; 25.5% for clindamycin and 6% for azithromycin (Table S3.1.1).

3.1.3.2 Estimation of PEC and PNEC

PEC values in raw sewage ranged between 0.02 μg L^{-1} (erythromycin) to 27 μg L^{-1} (amoxicillin) (Table 3.1.2). In treated wastewater, erythromycin and amikacin are expected to be detected at concentrations of a few ng L^{-1}, whereas the highest concentration was predicted for cefuroxime axetil (6.6 μg L^{-1}).

Comparison of PEC values with monitoring data that originated from Greece and other countries showed that for most antimicrobials (e.g sulfamethoxazole, cefaclor, norfloxacin, ciprofloxacin, doxycline, azithromycin, trimephoprim, clindamycin, levofloxacin, ofloxacin) the concentrations were comparable for both raw and treated wastewater (Golet et al. 2002; Botitsi et al., 2007; Zhang and Li, 2011; Ratola et al. 2012; Kosma et al., 2014). On the other hand, a much higher PEC value was observed for amoxicillin compared to the measured concentrations in raw wastewater (Zhang and Li, 2011; Ortiz de García et al. 2013). This difference is mainly due to the chemical properties of this compound and its trend to degrade abiotically after excretion. Specifically, amoxicillin has a β-lactam ring structure which is susceptible to cleavage by abiotic processes such as hydrolysis and photodegradation (Andreozzi et al. 2004; Xu et al. 2011). The consumption-based approach adopted in this study for estimating PEC does not take into account elimination of target compounds in sewerage system due to abiotic and/or biotic processes, and thus, for chemicals that are susceptible to abiotic degradation such as amoxicillin the here-used PECs are likely to represent a worst-case scenario.

PNEC values were calculated for acute and chronic toxicity using experimental published data (where available) and ECOSAR (Table 3.1.2). Amongst aquatic organisms, green algae seem to be much more sensitive to antimicrobials comparing to daphnids and fish (Tables S3.1.3, S3.1.4). Regarding acute toxicity, experimental ecotoxicity data for at least one aquatic species was available for less than 50% of target antimicrobials, while experimental

values have been published for all three aquatic organisms only for 5 antimicrobials (clarithromycin, sulfamethoxazole, erythromycin, ofloxacin, metronidazole).

Table 3.1.1 Average annual consumption data and excreted amounts of the most often used antimicrobials in Greece for years 2008 to 2010. (The estimated quantities are based on the amounts sold for home medication).

Antimicrobials	**Category**	**Sold medicine boxes[a] (Pieces/year)**	**Consumed amounts of target compounds (Kg/year)[b]**	**Excreted amounts of target compounds (Kg/year)[c]**
Amoxicillin	β-lactams	5059945	45719	22631
Clarithromycin	macrolides	2650520	19233	6736
Cefuroxime axetil	cephalosporin	1643560	10094	6056
Ciprofloxacin	quinolones	1529347	8376	4446
Cefaclor	cephalosporin	1505234	7968	5777
Clindamycin	lincosamide	1158019	1577	401
Azithromycin	macrolides	1082648	1806	108
Cefprozil	cephalosporin	1053185	5910	3842
Doxycycline	semi-synthetic tetracycline	914796	838	586

Metronidazole	imidazole	634089	6960	3480
Cefuroxime	cephalosporin	487658	381	229
Trimethoprim	bacteriostatic	303500	3224	1934
Norfloxacin	fluoroquinolone	290721	1588	476
Sulfamethoxazole	sulfonamides	264266	2111	201
Levofloxacin	fluoroquinolone	252710	686	263
Loracarbef	carbapenem	244748	721	663
Amikacin	aminoglycoside	236677	160	40
Netilmicin	aminoglycoside	192280	37	30
Erythromycin	macrolides	180636	485	19
Moxifloxacin	fluoroquinolone	156313	508	102
Ofloxacin	fluoroquinolone	107960	89	75
Cefadroxil	cephalosporin	58231	426	375
Ceftriaxone	cephalosporin	52901	91	60

Meropenem	carbapenem	4227	38	25
Total consumption and excretion		20064171	119026	58555

[a]Sum of injectable, oral and other medicines, [b]Based on equation 3.1.1 in the manuscript.

[c]Based on equation 3.1.2 in the manuscript.

For the compounds for which no experimental data was available, ECOSAR prediction model was used. However, in many cases, very high EC_{50} values (g/L) were estimated from the model. Therefore, there is need for additional laboratory experiments to enable a more objective assessment of the true effects of these compounds on aquatic organisms and to allow for comparing laboratory with predicted data. PNEC values of the studied antimicrobials ranged from below 0.01 μg L^{-1} (amoxicillin, clarithromycin, ciprofloxacin, levofloxacin) to several g L^{-1} (netilmicin, ceftriaxone, amikacin), indicating significantly different toxicity among target compounds. Regarding chronic toxicity, experimental data was available for only 8 out of 24 compounds, while ECOSAR does not allow a reliable estimation of NOEC or LOEC (Table 3.1.2, Table S3.1.4). As a result, a significant gap in the literature is observed, and there is insufficient knowledge regarding the chronic effects caused by most antimicrobial substances. For the compounds that chronic toxicity data was available, PNEC values ranged between 0.078 μg L^{-1} (amoxicillin, green algae) and 10,000 μg L^{-1} (trimephoprim, fish) (Table 3.1.2).

3.1.3.3 Environmental risk assessment

To predict the ecological threat due to the disposal of wastewater containing antimicrobials to the aquatic environment, RQ values were calculated for raw and treated wastewater for algae, daphnids and fish (Figure 3.1.1 and Tables 3.1.3, 3.1.4). Regarding acute toxicity, for 7 out of 24 target compounds (29%), RQ values higher than 1 were calculated for algae (Figure 3.1.1a,b and Table 3.1.3). Amongst them, amoxicillin, clarithromycin, ciprofloxacin and levofloxacin presented high risks with RQ values higher than 20, while RQ values between 1 and 10 were calculated for azithromycin, sulfamethoxazole and ofloxacin. Concerning the other aquatic organisms, only sulfamethoxazole presented a RQ slightly higher than 1 for daphnids and raw wastewater (Table 3.1.3), while no ecotoxicological risk was expected for fish due to the presence of target antimicrobials in raw and treated wastewater (Figure 3.1.1 a,b).The results of this study revealed that the high consumption of antimicrobials does not simultaneously indicate high environmental risk for the aquatic environment. For instance, beside the fact that cefaclor was one of the most often used antimicrobials (Table 3.1.1), its potential risk was estimated to be minimal for all aquatic organisms, giving RQ values equal to 0.0095, 0.0021 and 0.001 for algae, fish and daphnids, respectively (raw wastewater).

Table 3.1.2 Calculation of Predicted Environmental Concentration (PEC) in raw and treated wastewater and estimation of Predicted No-Effect Concentration (PNEC) for acute and chronic toxicity of target compounds.

	Raw Wastewater[a]	**Treated Wastewater[a]**		**Acute toxicity[b]**	**Chronic toxicity[b]**
Antimicrobials	**PEC (μgL^{-1})**	**PEC (μgL^{-1})**	**Test organism**	**PNEC (μgL^{-1})**	**PNEC (μgL^{-1})**
Amoxicillin	27	3.0	Fish	2544.97[c]	10
			Daphnid	1281.03[c]	n.c.
			Green algae	0.00222	0.078
Clarithromycin	8.1	4.5	Fish	12.21	n.c.
			Daphnid	25.72	n.c.
			Green algae	0.01	0.052
Ciprofloxacin	5.3	1.8	Fish	7285.35 [c]	2000
			Daphnid	3414.68 [c]	1200
			Green algae	0.01	n.c.

Cefaclor	6.9	0.43	Fish	7055.90[c]	n.c.
			Daphnid	3335.24[c]	n.c.
			Green algae	730.63[c]	n.c.
Cefprozil	4.6	3.6	Fish	3851.68[c]	n.c.
			Daphnid	1897.03[c]	n.c.
			Green algae	477.79[c]	n.c.
Azithromycin	0.13	0.07	Fish	11.27[c]	n.c.
			Daphnid	120.00	n.c.
			Green algae	0.02	n.c.
Metronidazole	4.2	2.6	Fish	6751.78[c]	200
			Daphnid	3051.89[c]	5000
			Green algae	38.80	n.c.
Norfloxacin	0.57	0.25	Fish	90144.03[c]	n.c.
			Daphnid	36063[c]	n.c.

			Green algae	10.40	40.2
Sulfamethoxazole	0.24	0.06	Fish	562.50	n.c.
			Daphnid	0.21	n.c.
			Green algae	0.03	n.c.
Erythromycin	0.02	0.001	Fish	0.94	n.c.
			Daphnid	22.45	n.c.
			Green algae	0.02	n.c.
Netlmicin	0.04	0.03	Fish	2.35+E06[c]	n.c.
			Daphnid	7.88+E05[c]	n.c.
			Green algae	53639[c]	n.c.
Loracarbef	0.80	0.62	Fish	5969.64[c]	n.c.
			Daphnid	963.00	n.c.
			Green algae	638.17[c]	130
Ofloxacin	0.09	0.05	Fish	0.53	250

			Daphnid	1.44	n.c.
			Green algae	0.02	0.1
Ceftriaxone	0.07	0.04	Fish	1020000[c]	n.c.
			Daphnid	362000[c]	n.c.
			Green algae	30360[c]	n.c.
Cefadroxil	0.45	0.35	Fish	16113[c]	n.c.
			Daphnid	7231[c]	n.c.
			Green algae	1328[c]	n.c.
Meropenem	0.03	0.01	Fish	166000[c]	n.c.
			Daphnid	6474[c]	n.c.
			Green algae	7355[c]	n.c.
Cefuroxime axetil	7.3	6.6	Fish	3420[c]	n.c.
			Daphnid	1730[c]	n.c.
			Green algae	470[c]	n.c.

Clindamycin	0.48	0.35	Fish	241000[c]	n.c.
			Daphnid	92500[c]	n.c.
			Green algae	10030[c]	n.c.
Doxyxycline	0.70	0.27	Fish	240[c]	n.c.
			Daphnid	140[c]	n.c.
			Green algae	60[c]	n.c.
Cefuroxime	0.27	0.21	Fish	21990[c]	n.c.
			Daphnid	9770[c]	n.c.
			Green algae	1740[c]	n.c.
Levofloxacin	0.32	0.18	Fish	20240[c]	n.c.
			Daphnid	8950[c]	n.c.
			Green algae	0.01	n.c.
Amikacin	0.05	0.004	Fish	5.98E+11[c]	n.c.
			Daphnid	9.37E+10[c]	n.c.

			Green algae	4.84E+08[c]	n.c.
Moxifloxacin	0.12	0.12	Fish	2380[c]	n.c.
			Daphnid	1210[c]	n.c.
			Green algae	340[c]	n.c.
Trimethoprim	2.32	1.63	Fish	1870[c]	10000
			Daphnid	92	600
			Green algae	80	2550

[a]Based on equation 3 in the manuscript

[b]Based on equations 4a and 4b (respectively) in the manuscript

[c]PNECs were calculated using EC_{50}/LC_{50} values from ECOSAR model; n.c.: not calculated

Similarly, no ecotoxicological risk was estimated for cefuroxime axetil, clindamycin, cefprozil, doxycycline and metronidazole, which also belong to the top ten consumed compounds for years 2008-2010. Having in mind that variability of PNEC among target antimicrobials was more than 8 orders of magnitude, while PEC values cover only 4 orders of magnitude (Table 3.1.2), it is obvious that PNEC is a more important factor for RQ calculation compared to PEC. Based on this, future studies for the monitoring of antimicrobials in the Greek environment should not solely rely on consumption data but to ecotoxicity data as well.

For chronic toxicity, RQ values higher than 1 were estimated for 3 out of 8 antimicrobials for which toxicity values were available. These compounds were amoxicillin, clarithromycin and ofloxacin (Table 3.1.4). Amoxicillin presented high risk for algae (raw and treated wastewater) and fish (treated wastewater); whereas clarithromycin presented high risk for algae and both types of wastewater. It is evident that the lack of experimental data for the chronic effects of most antimicrobials on aquatic organisms does not allow drawing a clear conclusion for their possible risks due to the existence of these compounds in wastewater.

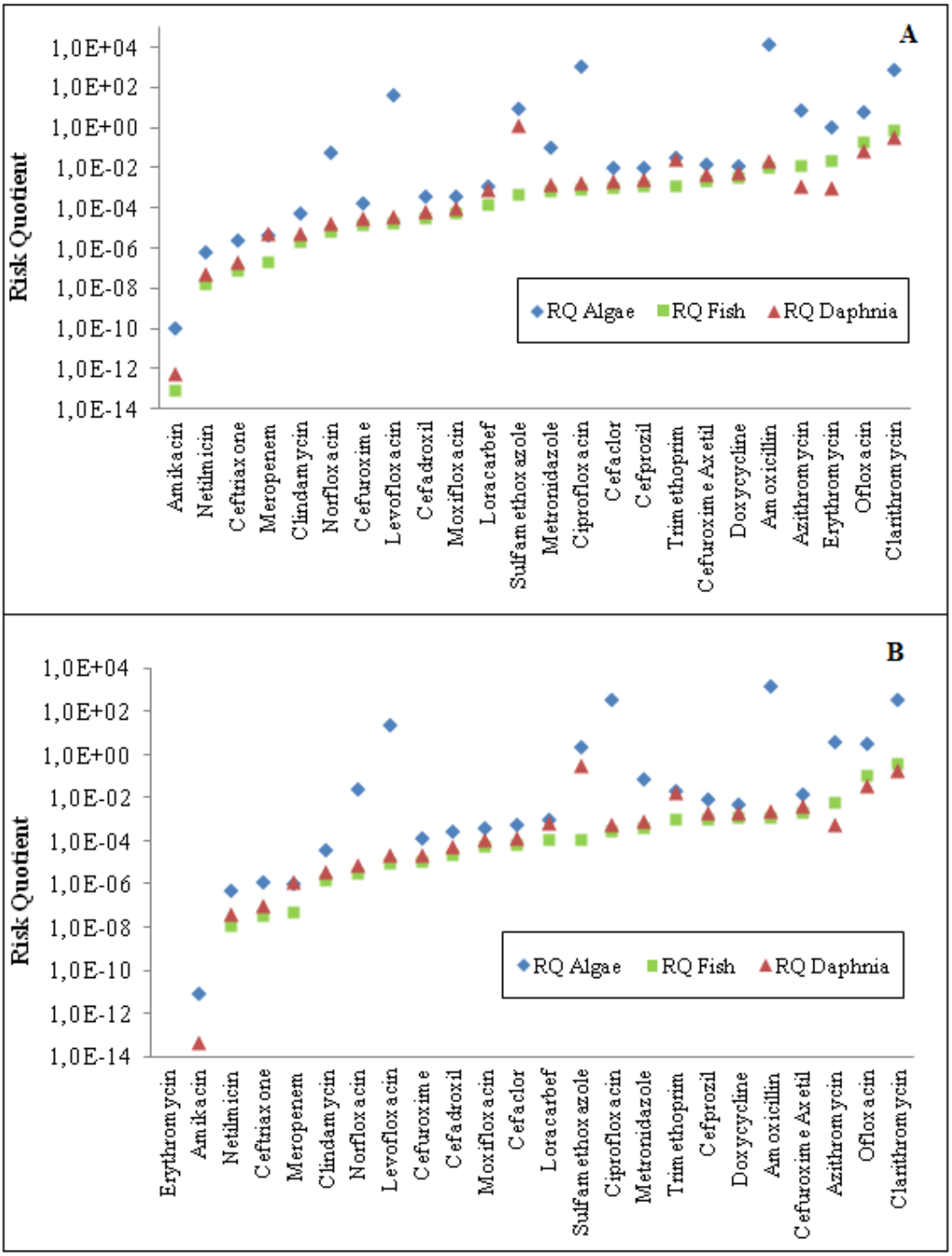


Figure 3.1.1 Risk Quotients of the Top-24 used antimicrobials in Greece ranked with increasing RQ in a) raw wastewater and (b) and treated wastewater. (RQs were calculated based on acute toxicity data for algae, daphnids and fish)

Table 3.1.3 Estimation of Risk Quotients (PEC/PNEC) for antimicrobials' acute toxicity (for all other antimicrobials RQ values were below 1)

Antimicrobials	Fish		Daphnids		Algae	
	Raw WW[a]	Treated WW	Raw WW	Treated WW	Raw WW	Treated WW
Amoxicillin	< 1	< 1	< 1	< 1	12243	1347
Clarithromycin	< 1	< 1	< 1	< 1	674	371
Ciprofloxacin	< 1	< 1	< 1	< 1	1068	363
Azithromycin	< 1	< 1	< 1	< 1	6.8	3.5
Sulfamethoxazole	< 1	< 1	1.1	< 1	8	2.1
Erythromycin	< 1	< 1	< 1	< 1	1	< 1
Ofloxacin	< 1	< 1	< 1	< 1	5.6	3.4
Levofloxacin	< 1	< 1	< 1	< 1	40	23

[a]WW: wastewater

Table 3.1.4 Estimation of Risk Quotients (PEC/PNEC) for antimicrobials' chronic toxicity (for all other antimicrobials RQ values were below 1)

Antimicrobials	Fish		Daphnids		Algae	
	Raw WW[a]	Treated WW	Raw WW	Treated WW	Raw WW	Treated WW
Amoxicillin	54	6	-	-	652556	328468
Clarithromycin	-	-	-	-	155	86
Ofloxacin	< 1	< 1	-	-	3.6	2.2

[a]WW: wastewater; (-): There is no toxicity data

Regarding acute mixture's toxicity, the level of environmental risk for all aquatic species was estimated to be low (Table 3.1.5). However, as it has been mentioned in the Materials and Methods section, mixture toxicity was estimated based on the hypothesis of concentration addition and for this reason baseline toxicities for each antimicrobial substance were calculated via the ECOSAR model (Escher et al. 2011).

Table 3.1.5 Estimation of antimicrobials mixture acute toxicity using ECOSAR.

Aquatic Organisms	**Risk Quotients**		**Environmental Risk**
	Raw WW[a]	**Treated WW**	
Fish	0.19	0.10	Low
Daphnia	0.28	0.14	Low
Algae	0.50	0.23	Low

[a]WW: wastewater

To investigate whether the use of baseline toxicity is appropriate or if there is a probability of underestimation of mixture toxicity as antimicrobials exhibit specific mode of toxic action to the organism, the Toxic Ratio (RT) was calculated according to Equation 3.1.7.

$$TR = \frac{EC_{50,baseline\_toxicity}}{EC_{50,experimental}} \quad (3.1.7)$$

According to Verhaar et al (1992), for TR > 10, the compound is likely to have a specific mode of toxic action, whereas if TR ≤ 10 it exhibits merely baseline toxicity. For 9 out of 10 compounds for which acute toxicity data was available for algae, TR values higher than 10 were calculated (Table 3.1.6). For instance, TR values as high as 217,626, 198,066 and 135,105 were obtained for ciprofloxacin, levofloxacin and amoxicillin, respectively. Moreover, TR values higher than 10 were obtained for sulfamethoxazole, ofloxacin and trimethoprim for daphnids, as well as for ofloxacin and erythromycin for fish (Table 3.1.6). Assuming similar results of TR analysis for the antimicrobials for which no experimental toxicity data was available, it is estimated that 90% of target antimicrobials present are likely

to exhibit a specific mode of action when present in mixtures and only 10% act as baseline toxicants. So far, several examples have been given in the literature for pharmaceuticals exhibiting specific mode of action such as genotoxicity, estrogen/androgen receptor binding, interference with photosynthesis on aquatic organisms (Neuwoehner et al., 2009; Margiotta-Casaluci et al., 2013). Based on the above, a higher risk than estimated here due to existence of these compounds in mixtures cannot be excluded. Future studies should be focused to estimation of toxicity of antimicrobial mixtures, and on the understanding of their mode of action on aquatic organisms.

Table 3.1.6. Toxic Ratios (TR) in fish, daphnia and algae for the target antimicrobials that experimental acute toxicity data was available.

Antimicrobials	**Toxic Ratio (TR)**		
	Fish	**Daphnia**	**Algae**
Amoxicillin	-	-	135105
Clarithromycin	5	2	2251
Ciprofloxacin	-	-	217626
Metronidazole	-	-	14
Norfloxacin	-	-	155
Sulfamethoxazole	7	8561	13870
Loracarbef	-	3	-
Erythromycin	237	6	3571
Ofloxacin	446	6216	97795
Trimethoprim	-	14	4
Levofloxacin	-	-	198066

(-): no experimental data available

To estimate the environmental risks associated with the occurrence of antimicrobials in Greek rivers, RQ values were calculated for algae considering the dilutions of discharged wastewater to receiving water bodies. Among 24 studied rivers receiving treated wastewater, 1 river presented a Dilution Factor (D) less than 10, in 8 rivers D was between 10 to 100, in 13 rivers D was between 100 to 1000 and in 2 rivers D was more than 1000 (Table S3.1.5). For rivers where D was less than 10, a high risk for 5 compounds was calculated with the highest RQ value of 449 for Amoxicillin (Table 3.1.7). Under conditions of medium dilution (D = 10-100), a high ecological threat was still expected for amoxicillin, clarithromycin and azithromycin, while lower risk was expected for rivers with D ranging between 100 and 1,000. Regarding chronic toxicity, only amoxicillin constituted a risk in rivers with low and medium dilution; whereas for the other compounds RQ values were lower than 1 independently of the dilution achieved.

Table 3.1.7 Estimation of Risk Quotients (PEC/PNEC) for antimicrobials' acute and chronic toxicity in algae for Greek rivers presenting different Dilution factors (D).

Antimicrobials	**RQ values for Algae**				
	Acute toxicity			**Chronic toxicity**	
	D<10	**D=10-100**	**D=100-1000**	**D<10**	**D=10-100**
Amoxicillin	449	85	4.8	29	5.4
Clarithromycin	126	23.3	1.3	< 1	< 1
Ciprofloxacin	29	5.4	< 1	-	-
Azithromycin	63	12	< 1	-	-
Sulfamethoxazole	3.7	< 1	< 1	-	-
Erythromycin	10	2.0	< 1	-	-
Ofloxacin	1.1	< 1	< 1	< 1	< 1
Levofloxacin	7.7	1.5	< 1	-	-

(-): There is no toxicity data

3.2 Simultaneous removal of nutrients and antimicrobials through human urine and treated wastewater cultivated with *Lemna minor* (Paper II)

3.2.1 Introduction

Constructed wetland technology is a promising alternative treatment process for removing conventional and non-conventional pollutants from wastewater (Stefanakis et al., 2011; Avila et al., 2014). Among different plant-based systems, duckweed ponds are of special interest as they achieve significant removal of major pollutants and heavy metals (Sekomo et al., 2012; Zhang et al., 2014). Recent studies have also reported the removal of emerging contaminants such as pharmaceuticals and personal care products in these systems due to several biotic and abiotic mechanisms (Reinhold et al. 2010; Zhang et al., 2014). Additionally, duckweeds can produce biomass with high crude protein content due to their ability to metabolize ammonia directly from water body (Mohedano et al., 2012), while they can accumulate high percentages of starch, a fact that allow their use for bioethanol production (Xu et al., 2011; Ge et al., 2012).

In domestic wastewater, 85% of the total N and 50% of the total P originate from human urine (HU), indicating that separately collected HU could be used for nutrients recovery and crop production (Liu et al., 2013; Zhang et al., 2013). When urine leaves the human body it contains urea, inorganic ions, natural organic metabolites as well as traces of antimicrobials and other synthetic organic chemicals that are related to health protection and human habits. Nonetheless, literature data for urine composition vary widely; the main characteristics of HU are: pH 5-8, urea 5000-9000 mg L^{-1}, NH_4^+-N 250-8100 mg L^{-1}, COD 8000-10000 mg L^{-1}, K^+ 1300-3100 mg L^{-1} and TP 350-2000 mg L^{-1} (Chang et al., 2013; Tuantet et al., 2014a; Zhang et al., 2013). It is worth mentioning that urine composition changes during transportation and storage, leading to an increase of pH and NH_4^+-N due to hydrolysis and a decrease of Mg due to precipitation of crystals. Regarding antimicrobials, parent compounds as well as their metabolites have been detected in HU at concentrations ranging up to some hundreds mg L^{-1}, depending on medical treatment (Gika et al., 2010; Cazola-Reyes et al., 2014).

In some recent studies, HU has been used for cultivating aquatic microorganisms in order to produce biomass that can be valorized as biofertilizer, biochemicals and biofuels. Tuantet et al. (2014a) studied the growth of *Chlorella sorokiniana* using different types of urine and in the presence of additional trace elements. Moreover, they achieved continuous cultivation of

these microalgae, producing biomass that contained up to 53% w/w and 25% w/w proteins and total fatty acids, respectively (Tuantet et al., 2014b). In another study, Zhang et al. (2014) used fresh urine to cultivate *Chlorella sorokiniana*, recovering in biomass 80.4% and 96.6% of N and P, respectively; while Chang et al. (2013) reported cultivation of *Spiroulina platensis* in HU under autotrophic and mixotrophic conditions, achieving significant NH_4^+-N, P and urea removal as well as high protein content. On the other hand, there is no information for the cultivation of duckweed using HU, as well as for the characteristics of produced biomass and the removal of nutrients and antimicrobials in such systems.

Based on the above, the main objective of this study was to investigate duckweed's *L. minor* growth using HU. Experiments were conducted using different types of urine (fresh, hydrolyzed, stored, and synthetic) and the effect of several parameters such as urine dilution, temperature, existence of macro- and microelements on growth rate was investigated. The efficiency of *L. minor* to remove nutrients (COD, total N, NH_4^+-N, total P) and selected antimicrobials (sulfamethoxazole, SMX and ciprofloxacin, CIP) from HU and treated domestic wastewater was also studied; while the content of produced biomass on protein and starch was determined.

3.2.2 Materials and Methods

3.2.2.1 Chemicals and culture

Analytical standards of SMX and CIP hydrochloride were purchased from Sigma – Aldrich (Steinheim, Germany). The physicochemical properties of two selected antimicrobials can be found in Table S3.2.1. Stock solutions were prepared in methanol (Fisher, USA). Culture of *Lemna minor L.*, clone St. was donated by Federal Environment Agency (Berlin, Germany). Before their use in urine and wastewater experiments, the duckweed cultures were grown for 4 weeks in Swedish standard (SIS) sterile growth medium (Table S3.2.2) according to the conditions described by OECD Guideline 221 (OECD, 2006). All salts used for *L. minor* growth medium were purchased by Fluka (Heidelberg, Germany). HPLC grade water was prepared in the laboratory using a MilliQ/MilliRO Millipore system (Bedford, USA). Regenerated Cellulose (RC) filters (0.2 μm, 4 mm) for antimicrobials analysis were purchased from Phenomenex (Torrance, CA, USA). HU and secondary treated wastewater used in this study were collected from the University Campus (Lesvos island, Greece).

3.2.2.2 Experiments with *L. minor*

Role of different parameters on *L. minor* growth rate

Experiments were initially conducted to investigate the optimal conditions for cultivating *L. minor* in urine. Different dilution factors (1:2, 1:5, 1:10, 1:25, 1:50, 1:100, 1:150, 1:200, 1:250) of HU and synthetic urine (SU) were tested and the growth rates of *L. minor* were calculated. HU was used in three different forms (fresh, hydrolyzed, stored for 1 day at 4 ^{o}C), while not hydrolyzed SU was prepared according to Table S3.2.3. Hydrolysis of HU was achieved by continuous mixing on a shaker for 30 min at 30^{o}C (Tuantet et al., 2014a). Experiments were also performed at different temperatures (12^{o}C, 18^{o}C, 24 ^{o}C and 30^{o}C), different initial mass of duckweed (0.5 gr, 1.0 gr and 1.5 gr) and in the presence of different macroelements (Fe, Ca, Mg) and mixture of microelements (B, Mn, Mo, Zn, Cu, Co). The experimental conditions used in each experiment are reported in Table 3.2.1.

All experiments were conducted in triplicate in glass petri dishes (12 cm diameter), containing 100 ml of each tested media. Each petri dish was inoculated with 12 healthy fronds of *L. minor* or appropriate mass of duckweed and incubated in a temperature-controlled incubator under continuous illumination with fluorescent lamps. The pH was adjusted to 7, using HCl or NaOH.

Nutrients and antimicrobials removal in *L. minor* experiments with urine and wastewater

Experiments with SIS medium, HU and secondary treated domestic wastewater were conducted in petri dishes to investigate the elimination of COD, urea, NH_4^+-N, TN and TP and the removal of two antimicrobials from different classes commonly found in HU (SMX and CIP) in the presence of *L. minor* (Table 3.2.2). These substances were chosen according to previous studies as two of the most often used antimicrobials in Greece (Iatrou et al., 2014) that are not totally removed during conventional wastewater treatment (Thomaidi et al., 2015). The duration of the experiments was 14 days and the tested concentration for antimicrobials was 50 μg L^{-1}. Before the addition of target antimicrobials, toxicity tests were conducted for a wide range of concentrations (SMX: 2-2000 μg L^{-1}; CIP: 50-450 μg L^{-1}) to investigate possible toxicity of these compounds to *L. minor*.

Table 3.2.1 Experimental protocol applied in *L. minor* growth rate experiments (number of replicates: 3).

Experiment	Type of Urine	Dilution Factor	Temperature (°C)	Initial number of leafs/Initial mass of duckweed (g)	pH	Duration (d)	Macro-, Micro elements
A	Fresh HU	1:2, 1:5, 1:10, 1:25, 1:50, 1:100, 1:150, 1:200, 1:250	24	12 leafs	7	7	No addition
	Hydrolyzed HU, Stored HU, SU	1:150, 1:200, 1:250					
B	Stored HU, SU	1:200	12, 18, 24, 30	12 leafs	7	7	No addition
C	Stored HU	1:200	24	0.5 g, 1 g, 1.5 g	7	7	No addition
D	Stored HU	1:200	24	0.5 g	7	10	Fe[1]
							Ca[2]
							Mg[3]
							B, Mn, Mo, Zn, Cu, Co[4]

[1]0.17 mg L^{-1}, [2]9.8 mg L^{-1}, [3]7.4 mg L^{-1}, [4]B: 0.17 mg L^{-1}, Mn: 0.056 mg L^{-1}, Mo: 0.004 mg L^{-1}, Zn: 0.011 mg L^{-1}, Cu: 0.013 mg L^{-1}, Co: 0.002 mg L^{-1}.

Table 3.2.2 Experimental protocol applied in *L. minor* experiments investigating nutrients and antimicrobials elimination (T: 24 ^{o}C; pH: 7; Duration: 14 d).

Experiment	Growth Medium	Initial mass of *Lemna minor* (g)	Harvesting	Antimicrobials ($\mu g\ L^{-1}$)
A[1]	SIS medium	1.5	No	50
B[2]	Stored HU (dilution 1:200)	1.5	No	50
C[2]	Stored HU (dilution 1:200)	1.5	Yes	50
D[2]	Treated wastewater	1.5	No	50
E	SIS medium	No addition of duckweed	No	50
F	Stored HU (dilution 1:200)	No addition of duckweed	No	50
G	Treated wastewater	No addition of duckweed	No	50

[1]*L. minor* acclimatized in medium SIS; [2]*L. minor* acclimatized to secondary treated domestic wastewater (acclimatization was conducted gradually in a period of 2 weeks).

Aqueous samples for the determination of nutrients and antimicrobials were taken at different time intervals, while biomass samples were taken at the beginning and at the end of the experiment to characterize duckweed for crude protein and starch content. To investigate the role of biomass harvesting on removal of nutrients and antimicrobials, experiments were also conducted with HU and harvesting of 0.5 g biomass at Days 5 and 10. To study the role of abiotic factors on the removal of antimicrobials, additional experiments were conducted in the absence of duckweed for all tested media (Table 3.2.2).

Starch accumulation in *L. minor* experiments with urine and wastewater

To study starch accumulation in duckweed, duplicate experiments were conducted using SIS medium, stored HU (dilution factor: 1:200) and secondary treated wastewater in petri dishes containing 100 mL of tested media, temperature of 24 ^{o}C, pH 7 and initial mass of duckweed equal to 1.5 g. The total duration of these experiments was 28 days and the concentration of starch was determined at Days 0, 7, 14, 21 and 28. As it has been reported in the literature that the starch content of duckweed may increase after its transfer in water containing no nutrients (Xu et al., 2011; Ge et al., 2012; Xiao et al., 2013), additional experiments were conducted with the aforementioned media. In these cases, 7 days after the start of the experiment, the cultures were transferred in petri dishes with tap water and kept there up to the end of the experiments.

3.2.2.3 Analytical methods

The determination of COD, NO_3^--N, TP and TN in aqueous samples was conducted according to Standard Methods (APHA-AWWA-WPCF, 2005). The urea determination was based to the modified diacetylmonoxime colorimetric assay (Mulvenna and Savidge, 1992; Rozet et al., 2007), while an Ion Chromatography system (ICS-3000, Dionex Co., Sunnyvale, CA, USA) with suppressed conductivity detection was used for the determination of NH_4^+-N and other cations (Na, K, Mg, Ca) for the characterization of HU composition. Prior to Ion Chromatography (IC) analysis, all samples were filtered (0.45 μm) and acidified for proper preservation. Starch content in duckweed samples was determined according to anthrone method (Hansen and Møller, 1975), while calculation of crude protein was based on the measurement of TN concentration in biomass (Xiao et al., 2013). Before the determination of starch and crude protein, the fresh biomass was dried overnight at 95 ˚C.

For the determination of target antimicrobials, aqueous samples were filtered through RC filters (0.2 μm, 4 mm), mixed with MeOH and analyzed in an HPLC system associated with a diode-array detector (DAD) (LC-20AD / SPD-M20A / CTO-20A / SIL-20A Shimadzu, Japan). Antimicrobials were separated from medium components using isocratic separation with aqueous 0.5% HCOOHin 0.05M CH_3COONH_4 : MeOH (70:30, v/v) at a flow rate of 1 mL min^{-1}. Chromatographic separation was achieved with a Zorbax reverse phase SB–C18 analytical column (150x4.6 mm; 5μm, Agilent) at 30 ˚C, using a guard column SB-C18. The acquisition wavelengths were 280 nm for CIP and 270 nm for SMX. The analytical procedure was based on a previously published method (Ašperger et al., 2009); the method limit of detection (LOD) was 364 ng L^{-1} for SMX and 1296 ng L^{-1} for CIP.

3.2.2.4 Calculations and data analysis

For the comparison of *L. minor* culture growth under different conditions, the specific growth rate in each condition was calculated against the culture grown in SIS medium (control). Specific growth rate was calculated by a linear regression of the natural logarithm versus culturing time, according to the following equation 3.2.1 (OECD, 2006; Gatidou et al., 2015):

$$\mu_{(i-j)} = \frac{\ln N_j - \ln N_i}{t} \quad (3.2.1)$$

Where, $\mu(i\text{-}j)$ is the average growth rate from time*i*to*j*, N_i and N_j are the corresponding biomass amount (g) or leaf number and t is the time period from i to j.

Specific growth rate normalized to area (μ_{area} as g m^{-2} d^{-1}) was calculated according to Equation 3.2.2 using fresh or dry weight mass data (Zhao et al., 2014; Xiao et al., 2013).

$$\mu_{area} = \frac{IW}{A \times t} \quad (3.2.2)$$

Where, IW is the average increased weight of dry or fresh biomass, A is the area of petri dish and t is the total time period of the experiment.

OriginPro 8 SR0 (Version 8.0724, OriginLab Corporation, Northampton, USA) was used for the construction of all graphs in current study. For the selection of best cultivation parameters, growth rates were checked with SPSS 21.0 by one-way ANOVA for the role of temperature and with two-way ANOVA for the type of urine, the dilution rate as well as of

the effect of the initial mass of *L. minor* in relation to the type of the substrate. A three-way ANOVA was used to examine the effects of time, type of substrate and the transfer of the cultures in water containing no nutrients. When ANOVA was significant at $p < 0.05$, the Tukey's HSD post hoc test was run to identify differences between treatments.

3.2.3 Results and Discussion

3.2.3.1 Role of different factors on *L. minor* growth

Experiments were conducted to investigate *L. minor* growth in urine. The characteristics of fresh HU are presented in Table 3.2.3 and were comparable to the values commonly found in the literature. According to leaf measurements, a growth rate of 0.33 d^{-1} was calculated for *L. minor* cultivated in SIS medium, whereas when urine was used the higher growth rates (0.20 to 0.24 d^{-1}) were observed for HU that had been diluted 200 times before the experiment (Figure 3.2.1). When smaller dilution rates were applied, a significant inhibition of duckweed growth ($p < 0.05$) was observed, ranging from 42% to 97% for dilution factors of 1:150 to 1:2, respectively. For dilution factor equal to 1:200, comparison among different types of HU types showed that the higher growth and the best characteristics of *L.minor*leafs (green and healthy) were observed for urine that had been stored for 1 d before use as well as for SU; slower growth and pallid leafs were observed in experiments with hydrolyzed HU. It is widely known that during hydrolysis urea is hydrolyzed by the enzyme urease to ammonia and carbamate (Udert et al., 2003; Liu et al., 2013; Tuantet et al., 2014a). The high concentrations of ammonia in hydrolyzed urine observed in this study (Table 3.2.3) could affect *L. minor* growth (Tuantet et al., 2014a; Xiao et al., 2013). HU stored for 1 d with dilution 1:200 were selected as the best medium for the following experiments with the highest growth rate ($p<0.01$).

To investigate the role of temperature on duckweed growth, experiments were conducted at four different temperatures in SIS, stored and synthetic HU. The two-way ANOVA showed that both temperature and type of substrate significantly affected the duckweed growth ($p < 0.05$), while their interaction was not significant. Specifically, for stored and synthetic HU, the highest growth rate was observed at 24 ˚C (Figure 3.2.2) with significant statistical differences ($p<0.001$). In experiments with stored HU and SU at temperature of 18 ^{o}C, the growth rate decreased by 40% comparing to 24 ˚C, while inhibition higher than 85% was

noticed at temperatures of 12 ºC and 30 ºC. According to the protocol describing the use of *L. minor* for toxicity tests (OECD, 2006), this organism can be maintained at lower temperatures (4-10 ºC); however the running of experiments at 24 ºC is proposed. Moreover, greater growth rates of duckweeds at temperatures ranging between 22.5 ºC and 27.5ºC have been reported by Xiao et al. (2013).

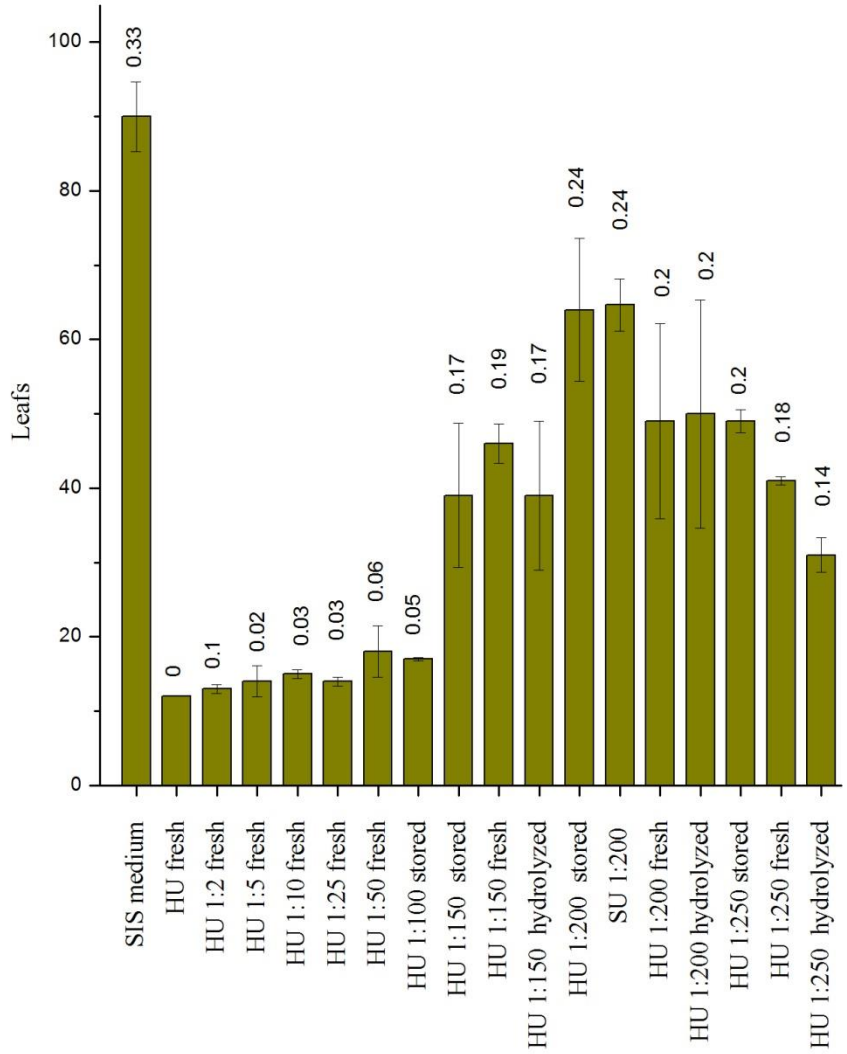


Figure 3.2.1 Effect of urine type and dilution factor on number of leafs and growth rate, μ (as d^{-1}) of *L. minor*. The values above each column represent calculated growth rates (duration of the experiment: 7 d; 12 leafs initial frond number; temperature: 24˚C; pH 7; growth rates were calculated using Equation 3.2.1).

Table 3.2.3 Characteristics of fresh, hydrolyzed and stored human urine (HU) used in this study and typical synthesis for fresh HU reported in the literature.

Parameter	**Fresh HU[1]**	**Fresh HU[2]**	**Hydrolyzed HU[1]**	**Stored HU[1]**
pH	6.35 ± 0.1	5 – 8	8.5 ± 0.3	7 ± 0.1
Conductivity, mS cm^{-1}	10 ± 0.2	6 - 23	19 ± 0.1	11 ± 0.1
Urea, mg L^{-1}	8000 ± 200	5000 – 9000	3660± 200	8000 ± 200
TP, mg L^{-1}	1020 ± 57	350 – 2000	800± 20	1100± 20
TN, mg L^{-1}	6000 ± 120	4000 – 10000	5400± 80	4200± 120
NO_3^-N, mg L^{-1}	280 ± 11	-	-	-
NH_4^+-N, mg L^{-1}	486 ± 16	250-8100	1276± 60	572± 20
COD, mg L^{-1}	9000 ± 155	8000 - 15000	4000± 200	8000± 160
Na, mg L^{-1}	3000 ± 90	1800 – 5800	3200± 20	3000± 40
K, mg L^{-1}	3040 ± 14	1300 – 3100	2000± 18	3000± 16
Mg, mg L^{-1}	186 ± 8	29 – 121	24± 4	60± 4
Ca, mg L^{-1}	252 ± 11	96 – 233	300± 80	220± 8

[1]Data from current study; [2]Chang et al., 2013; Zhang et al., 2013; Tice et al., 2014; Tuantet et al.2014

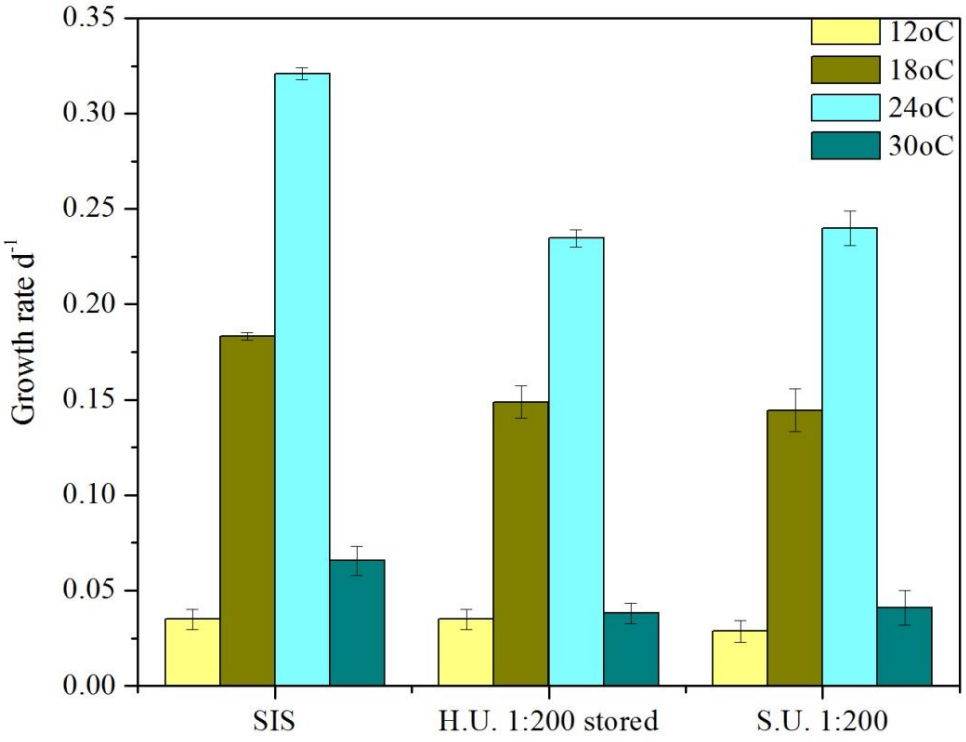


Figure 3.2.2 Growth rate values, μ (d^{-1}) from leaf measurements for tested temperatures in stored human urine (HU) and synthetic urine (SU) compared with control medium SIS (duration: 7 d; 12 leafs initial frond number; temperature: 24˚C; pH adjust to 7; growth rates were calculated using Equation 3.2.1)

The role of micronutrients and macronutrients on duckweed growth was investigated in experiments conducted with stored HU and dilution 1:200 as described in Table 3.2.1. No improvement on *L. minor* growth rate was noticed in the presence of micro/macronutrients (Figure S1). In a previous study (Xiao et al., 2013), the addition of micronutrients increased growth rate of different duckweed species (*Spirodela polyrhiza* (L.), *Lemna aequinoctialis* P1, *Landoltia punctata* S3, *La. punctata* OT); whereas to the best of our knowledge no data is available for the role of micronutrients or added macronutrients on *L. minor* growth.

The role of initial duckweed mass on growth rate was investigated using HU and SIS medium. Three different initial masses of *L. minor* (0.5 g, 1.0 g, 1.5 g) were tested and the specific growth rate, μ, as well as specific growth rate normalized to area, μ_{area}, were calculated (Table 3.2.4). According to the statistical analysis, both the initial mass of duckweed and the type of substrate affected μ and μ_{area} ($p < 0.001$). Specifically, the highest growth rates were observed at initial mass of duckweed equal to 1.5 g, while under these experimental conditions, both μ and μ_{area} were higher in HU than in the control experiment with SIS medium. For that reason, the initial mass of 1.5 g was chosen for the following experiments. It should be mentioned that the selected plant density mimicked the full surface coverage growth observed in natural and treatment wetlands (Trond and Saunders, 2006; Reinhold et al. 2010). According to the literature, for a specific range of plant densities, when more fronds initially exist greater biomass production can be achieved. On the other hand, the application of very high plant densities can inhibit duckweeds growth due to overcrowding phenomena (Xu, et al., 2011; Xiao et al., 2013).

Table 3.2.4 Role of initial amount of *L. minor* on production of biomass (g), specific growth rate, μ (d^{-1}) and specific growth rate normalized to area, μ_{area} (duration of the experiment: 14 d; temperature: 24˚C; pH: 7)

Parameters	**Initial Amount of *Lemna minor* (g)**		
	0.5	1.0	1.5
SIS Medium			
Duckweed mass (g) (Day 14)	1.41 ± 0.05	2.88 ± 0.1	4.73 ± 0.4
Growth rate, μ (d^{-1})[1]	0.074 ± 0.003	0.076 ± 0.003	0.082 ± 0.006
Growth rate, μ_{area} (g m^{-2} d^{-1})[2]	0.089 ± 0.003	0.182 ± 0.007	0.299 ± 0.026
Human urine			
Duckweed mass (g) (Day 14)	1.60 ± 0.05	3.60 ± 0.1	5.83 ± 0.35
Growth rate, μ (d^{-1})[1]	0.083 ± 0.002	0.091 ± 0.002	0.097 ± 0.04
Growth rate, μ_{area} (g m^{-2} d^{-1})[2]	0.101 ± 0.003	0.227 ± 0.006	0.369 ± 0.022

[1]calculated according to Equation 3.2.1 and using data of dry biomass; [2]calculated according to Equation 3.2.2.

3.2.3.2 Removal of nutrients in experiments with *L. minor*

The removal of major pollutants in experiments with stored HU and treated wastewater is presented in Figure 3.2.3 and Table S3.2.4. According to the results, *L. minor* efficiently removed COD and nutrients from HU and treated wastewater. Specifically, in experiments with HU, very high (>95%) COD and TP removal was achieved up to the end of the experiment (14 d), whereas the removal of urea, TN and NH_4^+-N was higher than 83%, 50% and 55%, respectively. Similar results were found when biomass was harvested during the experiment. High nutrients removal was also observed in experiments with treated wastewater, ranging from 70% to 100% for TN and NH_4^+-N, respectively. The preference of *L. minor* to remove nitrogen in the form of ammonia has been reported in the literature (Ge et al., 2012; Chang et al., 2013). Concerning TP, the results of the current study are similar or even higher compared to those reported in the literature for other tested media (Xu et al., 2011; Xu and Shen, 2011).

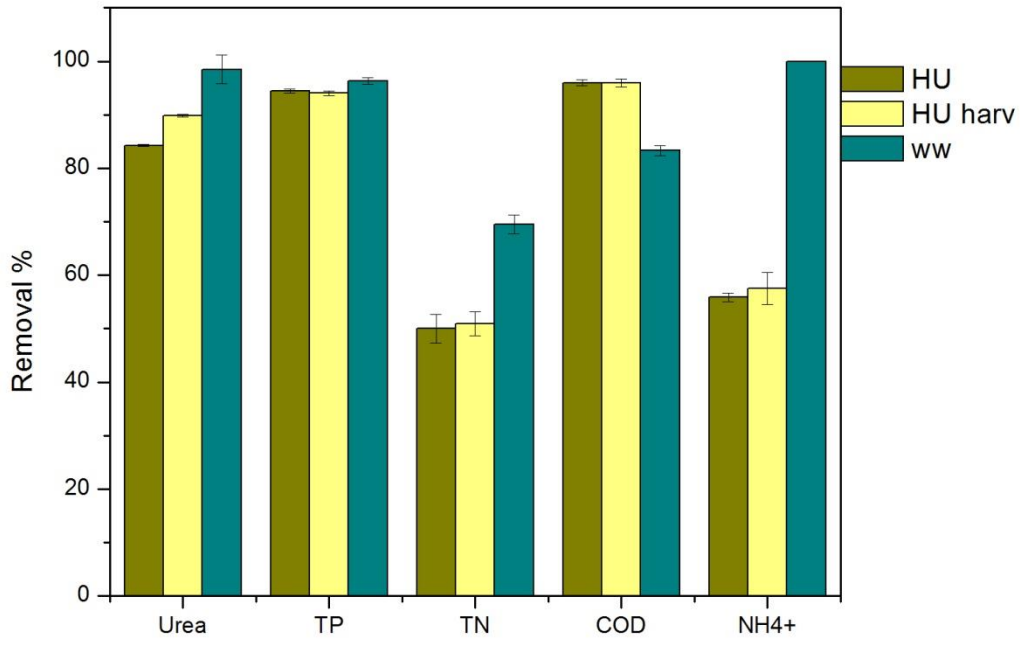


Figure 3.2.3 Removal of urea, TP, TN, COD and NH_4^+-N in experiments with human urine (HU), human urine and harvesting of biomass (HU harv) and secondary treated wastewater (ww) (duration: 14 d; initial mass of duckweed: 1.5 gr; temperature: 24˚C; pH: 7)

The changes in concentrations of urea and NH_4^+-N during the experiment are shown in Figure S3.2.2 and Table S3.2.4. Urea is hydrolyzed into NH_4^+-N prior its assimilation and hence the concentration of NH_4^+-N was found to increase at the beginning of the cultivation period (Figure S3.2.2). Similar trends for the concentrations of urea and NH_4^+-N have been reported in previous studies, investigating the cultivation of *Spiroulina platensis* in HU (Chang et al., 2013).

3.2.3.3 Removal of antimicrobials in experiments with *L. minor*

The elimination of SMX and CIP was investigated in abiotic and biotic experiments with SIS medium, HU and secondary treated wastewater. Before adding the antimicrobials in petri dishes with duckweed, their toxicity on *L. minor* was tested for a wide range of concentrations (Figure S3.2.3). CIP greatly affected *L. minor* growth and leafs' characteristics (yellow leafs with chlorosis) at concentrations equal or higher than 150 μg L^{-1}, while no considerable effect was noticed for SMX at concentrations up to 2000 μg L^{-1}. Based on the above, no toxic effects on *L. minor* are expected for the concentrations used in the current study (50 μg L^{-1}).

During the 14 d of the experiment, in the absence of *L. minor*, SMX was removed by a factor of 10% from SIS medium and HU and by 30% in the presence of treated wastewater (Figure 3.2.4A1). On the other hand, an almost total removal was observed for CIP under abiotic conditions up to the end of experiment (Figure 3.2.4B1). This removal of CIP is probably due to photodegradation as according to the literature this compound is very photosensitive (Girardi et al., 2011; Babić et al., 2013). The presence of duckweed improved significantly the removal of SMX, exceeding 80% in all tested media up to the end of the experiment (Figure 3.2.4A); whereas CIP removal was slightly decreased comparing to abiotic experiments (Figure 3.2.4B). These results indicate the role of plant uptake and bacterial activity on removal of SMX, while the deceleration of CIP removal could be due to the prevention of light penetration in the presence of duckweed.

The aforementioned results indicate the potential efficiency of systems with duckweeds to remove antimicrobials. Further experiments should be conducted with *L. minor* to evaluate the role of different mechanisms such as photodegradation, hydrolysis, biodegradation and plant uptake on the removal of antimicrobials and to identify the transformation by-products.

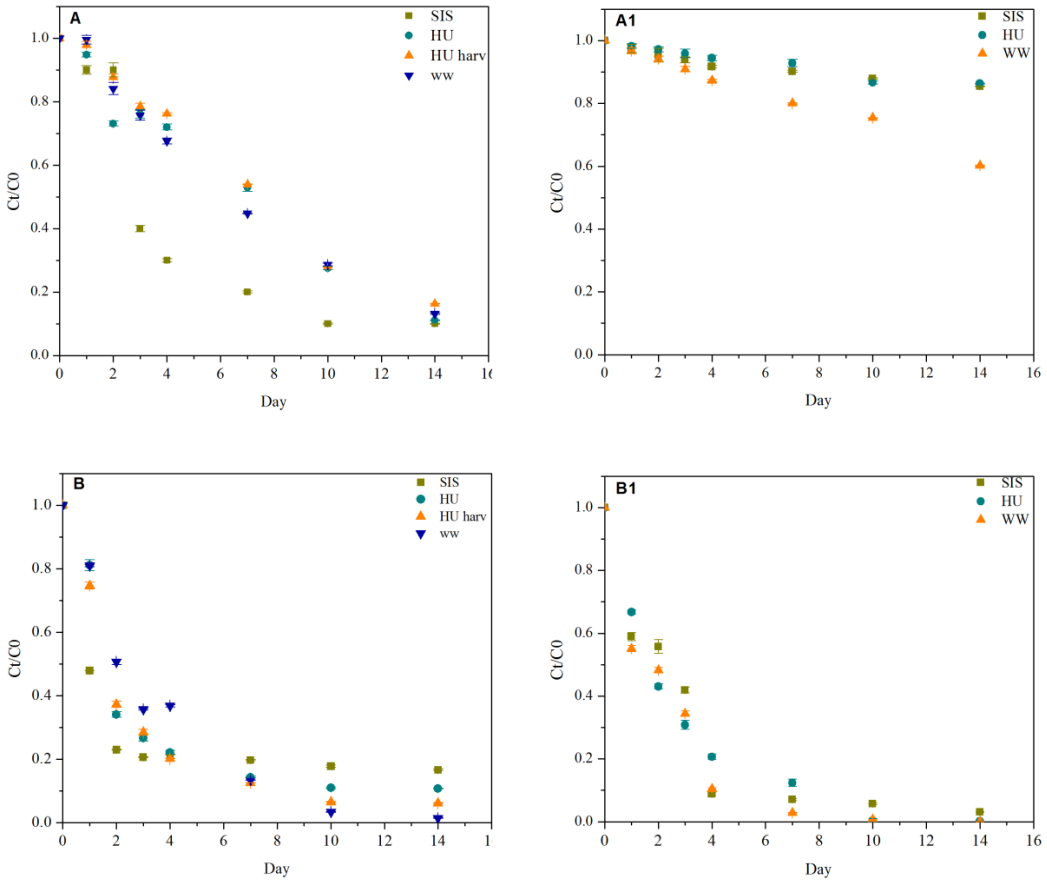


Figure 3.2.4 Removal of sulfamethoxazole, SMX (A) and ciprofloxacin, CIP (B) in experiments with *L. minor* cultivated in different tested media and removal observed under abiotic conditions, SMX (A1) and CIP (B1) (duration: 14 d; initial mass of duckweed: 1.5 g; temperature: 24˚C; pH: 7; SIS: control medium; HU: human urine; HU harv: human urine with harvested biomass at Days 5 and 10; WW: secondary treated wastewater)

3.2.3.4 Starch accumulation and crude protein in *L. minor* experiments with urine and wastewater

The content of *L. minor* in crude protein and starch at the start and at the end (14 d) of experiments with SIS medium, HU and treated wastewater is shown in Table 3.2.5. According to the results, the highest protein content (31.6%) was observed in experiments with HU and harvesting of biomass, whereas the highest starch content in experiments with treated wastewater (33.8%). The levels of crude protein from current study are comparable to

previous studies conducted with different media such as diluted swine and lagoon wastewater where the crude protein ranged between 10 % and 40 % (Mohedano et al., 2012; Xu et al., 2011; Xiao et al., 2013).

Table 3.2.5 Crude protein and starch content of *L. minor* cultivated in SIS medium, human urine (HU) and secondary treated wastewater (duration: 14 d; initial mass of duckweed: 1.5 g; temperature: 24˚C; pH: 7)

	Crude Protein (%)[1]			
Days	**SISmedium**	**Human urine**	**Human urine harvested**	**Treated wastewater**
0	21.82	25.32	25.32	25.32
14	23.29	28.76	31.60	25.26
	Starch (%)[2]			
0	17.7	21.0	21.0	21.0
14	26.7	31.8	28.85	33.8

[1]value of one sample per media; [2]mean value of two samples per media

It is known that fronds are the dominant starch storage organ, while there is a negative relationship between growth rate of the duckweed and starch storage (Ge et al., 2012; Xiao et al., 2013). To achieve higher starch accumulation an additional experiment took place for 28 d in the absence and presence of nutrients, as described in session 3.2.2.2. According to the statistical analysis of the results summarised in Table 3.2.6, the 'time' (that is the day of the determination), the 'type of substrate' (SIS, HU, treated wastewater) and the 'transfer' of the cultures in water containing no nutrients all exerted significant effects (at the 0.001 level) on the starch content. The post hoc tests revealed that the starch content increased significantly in all tested media, regardless the presence or absence of nutrients, up to the 21st d of the experiment ($p < 0.001$), while it remained constant up to the end of the experiment (28th d) ($p > 0.05$ between 21st and 28th d). The starch accumulation followed the order SIS < treated wastewater < HU ($p < 0.01$), whereas in the experiments conducted in the absence of nutrients (transportation of duckweed cultures in tap water at day 7), higher starch content was recorded than in treatments in media containing nutrients ($p < 0.001$) for all the examined substrates. Summing up the all the above, it follows that the highest starch content

was reached by duckweed cultivated for at least 21 d in HU after transfer to tap water at Day 7. As it has been previously mentioned, the deficiency of nutrients (N and P) helps duckweeds to faster accumulate starch (Xiao et al., 2013). The starch content achieved in this study is comparable to other studies conducted with agricultural and swine wastewater and where starch content in the range of 12.5% to 52.9% has been reported (Ge et al. 2012; Xu et al., 2011; Xiao et al., 2013).

Table 3.2.6 Starch content (%) in *L. minor* cultivated in SIS medium, human urine and treated wastewater (duration of the experiments: 28 d; initial mass of duckweed: 1.5 g; temperature: 24˚C; pH: 7)

Sample/Day	**0**	**7**	**14**	**21**	**28**
SIS medium[1]	17.9 ± 0.0	18.8 ± 0.1	26.1 ± 0.1	31.0 ± 0.0	31.4 ± 0.1
Human Urine[1]	19.9 ± 0.1	24.6 ± 0.1	30.4 ± 0.2	40.1 ± 0.1	40.9 ± 0.1
Wastewater[1]	19.9 ± 0.1	23.9 ± 0.0	28.2 ± 0.2	37.1 ± 0.1	37.8 ± 0.1
SIS medium[2]	17.3 ± 0.1	19.7 ± 0.1	28.4 ± 0.1	37.3 ± 0.1	38.6 ± 0.1
Human Urine[2]	19.9 ± 0.1	24.3 ± 0.0	34.3 ± 0.1	46.1 ± 0.0	47.1 ± 0.1
Wastewater[2]	19.9 ± 0.1	24.5 ± 0.1	31.9 ± 0.0	45.9 ± 0.0	43.4 ± 0.1

[1]all media remained the same until the end of the experiment; [2]cultures were transferred in dishes with tap water 7 d after the start of the experiment.

3.3 Investigation of biomass production, crude protein and starch content in laboratory wastewater treatment systems planted with *Lemna minor* and *Lemna gibba* (Paper III)

3.3.1 Introduction

Constructed wetlands planted with duckweeds have been widely used for removing conventional and non-conventional pollutants in secondary and tertiary wastewater treatment (Zhang et al., 2014; Reinhold et al., 2010; Priya et al., 2012; Iatrou et al., 2013). These aquatic plants belong to the *Lemnaceae* family (Table S3.3.1) and they are living in standing water or water with low flow. They have the advantage of being the smallest angiosperms in the world with rapid multiplication rate (doubling time: 48-72 h), while they are characterised by ease of harvest and inexpensive growth requirements (Gatidou et al., 2015). Furthermore, duckweeds can be found throughout the world except in the Arctic and Antarctic areas (Verma et al., 2015).

The duckweeds had been primarily used for the treatment of different types of wastewater, the elimination of heavy metals from contaminated waters as well as toxicity testers (Matos et al., 2014; Mohedano et al., 2012).However,recent studies have shown that the cultivation of duckweeds in different types of wastewater resulted to high amounts of biomass production with high crude proteinand starch contentdue to their ability to metabolize ammonia directly from water depending on harvesting time and nutrient content (Verma et al., 2015; Matos et al., 2014; Mohedano et al., 2012). The protein content in some types of duckweeds has been reported to range between 15% and 45% dry weight, depending on the cultivating and growing conditions (Mohedano et al., 2012; Zhao et al., 2015; Xu et al., 2011c). Additionally, the fronds of duckweeds are the dominant starch storage organ; whereas there is a negative relationship between duckweeds' growth rate and starch storage (Ge et al., 2011 and Xiao et al., 2013). Specifically, it seems that the deficiency of nutrients (N and P) helps duckweeds to faster accumulate starch (Ge et al., 2011). Under this frame, recent studies reported that the starch content of duckweeds cultivated in agricultural and swine wastewater was in the range of 12.5% to 52.9% (Ge et al., 2011; Xiao et al., 2013; Wang et al., 2014; Xu et al., 2011b).

So far, there is limited knowledge on the cultivation of different duckweeds in secondary treated domestic wastewater. In a previous study, Iatrou et al. (2015) used *L.minor* for urine and treated domestic wastewater treatment and reported that protein and starch content of the produced biomass reached 25.3% and 47.1%, respectively, under specific experimental

conditions. Garcia et al. (2017) used a duckweed pond with *Landoltia punctate* for polishing a stabilization pond effluent and reported that biomass production rate ranged between 3.6 and 10.3 g per m^2 and day in dry mass. de Matos et al. (2014) conducted outdoor experiments using effluents from facultative ponds and reported that the crude protein and the fiber content of the produced biomass was 38.03% and 16.17%, respectively.Toyama et al.(2018) cultured four different duckweeds (*S. polyrhiza*, *L. minor*, *L. gibba*, *L. punctata*) in treated municipal wastewater and other types of wastewater and reported that *S. polyrhiza* showed the higher biomass production and nitrogen removal for all types of wastewater. To the best of our knowledge, so far, the simultaneous cultivationof different duckweeds in treated domestic wastewater for biomass production with high crude protein and starch content has not been investigated.It is worth mentioned that a biomass with high protein content could be used as fertilizer oranimal feed for cattle and fish. On the other hand,the accumulation of starch in high percentages allows the potential use of duckweeds for the production of bioethanol, as starch can be easily saccharified to glucose.

Based on the above, the main objective of the current study was to investigate simultaneous duckweeds' cultivation using secondary treated domestic wastewater for producing biomass with high crude protein and starch content. For this reason, three lab-scale wastewater treatment systems were used. The 1st system was planted with *L.minor*, the 2nd with *L.gibba* and the 3rd with the combination of the two duckweeds. Each experiment divided in three experimental phases, in Phase A treated wastewater was used as substrate, in Phase B 30 mg L^{-1} NH_4-N were added to treated wastewater, while in Phase C duckweeds were grown in absence of nutrients. During the experiments, the crude protein and the starch content of biomass was investigated, as well as the ability of duckweeds to remove selected nutrients (NH_4-N, TP, TN) from wastewater.

3.3.2 Materials and Methods

3.3.2.1 Chemicals and Cultures

Culture of *Lemna minor L.,* clone St. was donated by Federal Environment Agency (Berlin, Germany). Before its use in wastewater experiments, the duckweed culture was grown for 4 weeks in Swedish standard (SIS) sterile growth medium (Table S3.3.2) according to the conditions described by OECD Guideline 221 (OECD, 2006). All salts used for *L.minor*

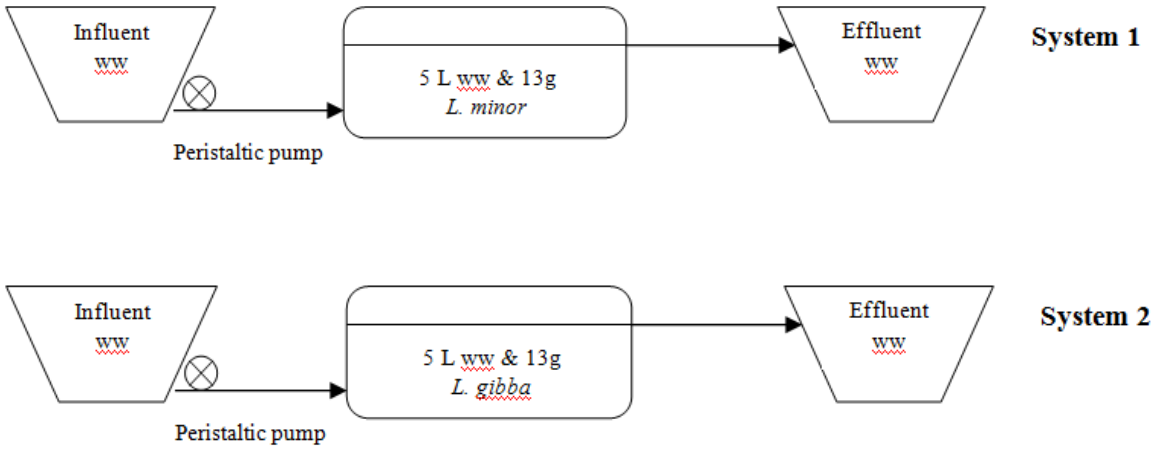
growth medium were purchased by Fluka (Heidelberg, Germany). HPLC grade water was prepared in the laboratory using a MilliQ/MilliRO Millipore system (Bedford, USA). Afterwards, the culture of *L.minor* was acclimatized for 6 weeks with secondary treated wastewater.

The culture of *Lemna gibba L.* was collected from the island of Lesvos, within Natura Area (GR4110012, North Lesvos), in a natural wetland at an altitude of about 400 meters. *L.gibba* was acclimatized for 6 weeks in tanks with secondary treated wastewater.

Secondary treated wastewater used in the current study was collected from the University Campus Sewage Treatment Plant (STP). This STP consists of a nitrifying activated sludge bioreactor and a secondary clarifier.

3.3.2.2 Experimental setup

Experiments were performed using secondary treated wastewater (Table 3.3.1) and two photosynthetic organisms belonging to duckweeds (*L.minor* and *L.gibba*) in a temperature-controlled room. The continuous flow set-up comprised from three parallel treatment lines with duckweed planted mini ponds (Figure 3.3.1); System 1 contained *L. minor*, System 2 contained *L. gibba*, while System 3 contained *L. minor and L. gibba*. Each pond had a working volume of 5 L and duckweed biomass was harvested every week in order to maintain the initial added biomass of 13 g during the experiment.



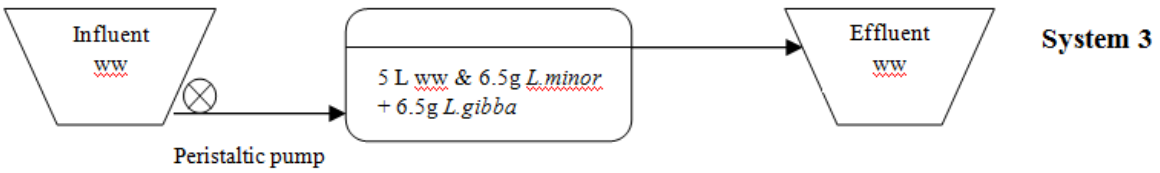


Figure 3.3.1 Lab-scale continuous-flow systems used in the current study (System 1: *L. minor*; System 2: *L. gibba*; System 3: *L. minor* + *L. gibba*). The systems received secondary treated wastewater (ww).

All systems operated at a hydraulic retention time (HRT) of 4 days under continuous light conditions (fluorescent lamps Philips, TLD 36 W/840, emission at 320–740 nm) and the flow rate was set at 0.87 ml min^{-1}. No evapotranspiration losses were observed. The experiments lasted 32 days and each one divided into two phases (Table S3.3.3). During Phase A, secondary treated wastewater was used as feed, while in Phase B an amount of ammonium nitrogen was added to wastewater in order to achieve initial concentration of 30 mg L^{-1} NH_4-N.

The monitoring of experimental systems was conducted via daily measurements of pH, T and flowrates. In a weekly basis, aqueous samples from the inlet and outlet of the systems were taken for chemical oxygen demand (COD), ammonium nitrogen (NH_4-N), total nitrogen (TN) and total phosphorus (TP) monitoring, while biomass samples were also taken for the determination of crude protein and starch content.

Table 3.3.1 Characteristics of secondary treated wastewater used in experimental Phases A and B in System 1 (*L. minor*), System 2 (*L. gibba*) and System 3 (*L. minor* + *L. gibba*)

Parameter	***System 1***		***Systems 2 and 3***	
	Phase A[1]	*Phase B*[2]	*Phase A*[1]	*Phase B*[2]
pH	7.5 ± 0.2^{a}	7.2 ± 0.3^{a}	7.9 ± 0.3^{b}	7.8 ± 0.2^{b}
COD (mg/L)	19.2 ± 6.3^{a}	19.2 ± 6.3^{a}	21.3 ± 3.0^{a}	21.3 ± 3.0^{a}
NH_4-N (mg/L)	0.3 ± 0.1^{a}	31.9 ± 2.9^{b}	1.7 ± 0.1^{c}	27.7 ± 3.5^{b}

NO_3-N (mg/L)	6.3 ± 1.8[a]	5.8 ± 2.3[a]	5.5 ± 0.8[a]	5.9 ± 1.1[a]
TP (mg/L)	1.2 ± 0.2[a]	1.3 ± 0.2[a]	1.2 ± 0.2[a]	1.4 ± 0.2[a]
TN (mg/L)	41.3 ± 2.2[a]	69.5 ± 2.6[b]	38.3 ± 2.2[a]	69.0 ± 3.2[b]

[1]*Phase A*: No addition of NH_4-N, [2]*Phase* B: Addition of 30mg/L NH_4-N

[2]*a, b, c*: different letters indicate statistical differences at 95% confidence level between Systems 1, 2 and 3

After the completion of Phase B, starch accumulation experiments were conducted in triplicates by using 100 mL tap water in petri dishes and initial masses of tested duckweeds equal to 2 g (Table S3.3.4). The total duration of those experiments was 21 days and the starch content was determined at Days 0, 7, 14 and 21.

3.3.2.3 Analyses and calculations

Analyses of COD, NH_4-N, NO_3-N, TP and TN were conducted according to Standard Methods (APHA, 2005). Starch content in duckweed samples was determined according to anthrone method (Hansen and Møller, 1975), while calculation of crude protein was based on the measurement of TN concentration in biomass (Xiao et al., 2013). Before the determination of starch and crude protein, the fresh biomass was dried overnight at 95 ˚C.

The specific growth rate was calculated by linear regression of the natural logarithm versus culturing time ($\mu_{(i-j)}$ as d^{-1}), according to OECD 221 protocol and the Equation 3.3.1 (Xu et al., 2011b):

$$\mu_{(i-j)} = \frac{\ln N_j - \ln N_i}{t} \quad (3.3.1)$$

Where, $\mu(i\text{-}j)$ is the average growth rate from time*i*to*j*, N_i and N_j are the corresponding biomass amount (g) or leaf number and t is the time period from i to j.

Furthermore, the specific growth rate normalized to area (μ_{area} as g m^{-2} d^{-1}) was calculated according to Equation 3.3.2 using fresh weight mass data (Xiao et al., 2013 and Iatrou et. al, 2015):

$$\mu_{area} = \frac{IW}{A \times t} \quad (3.3.2)$$

Where, *IW* is the increased wet weight of fresh biomass, *A* is the area of the tank and *t* is the time period of cultivation.

The calculation of the removal efficiency of each tested parameter was calculated according to the Equation 3.3.3:

$$Removal\ (\%) = (\frac{C_{in}-C_{out}}{C_{in}}) \times 100 \qquad (3.3.3)$$

Where, C_{in} is the concentration at the inlet (mg L^{-1}) and C_{out} the concentration at the outlet of each System (mg L^{-1}).

The results of current study were statistically checked with SPSS 17.0 by one-way ANOVA and paired-samples T-test. When ANOVA was significant at $p<0.05$, the Tukey's HSD post hoc test was run to identify differences between the tested parameters. OriginPro 8 SR0 (Version 8.0724, OriginLab Corporation, Northampton, USA) was used for the construction of figures.

3.3.3 Results and Discussion

3.3.3.1 Removal of nutrients in different experimental systems

The wastewater flow rate in all systems was similar, ranging between 0.86 ± 0.12 mL min^{-1}, 0.89 ± 0.17 mL min^{-1} and 0.89 ± 0.16 mL min^{-1} for System 1 (*L.minor*), System 2 (*L.gibba*) and System 3 (combination of two duckweeds), respectively. In System 1 that operated in March 2015, the average tank temperature was 19.9 ± 0.8 °C and the pH was slightly increased from 7.4 ± 0.3 in the influents to 7.8 ± 0.5 in the effluents. In Systems B and C that operated in April-May 2015, the average tank temperature was slightly higher reaching 21.7 ± 2.3 °C and the pH was increased from 7.9 ± 0.3 in the influents to 8.3 ± 0.6 in the effluents. Figure 3.3.2 shows the daily variation of temperature and pH in the experimental systems during Phase A and B. The 5% pH increment in the effluents was due to the photosynthetic activity of the duckweeds.

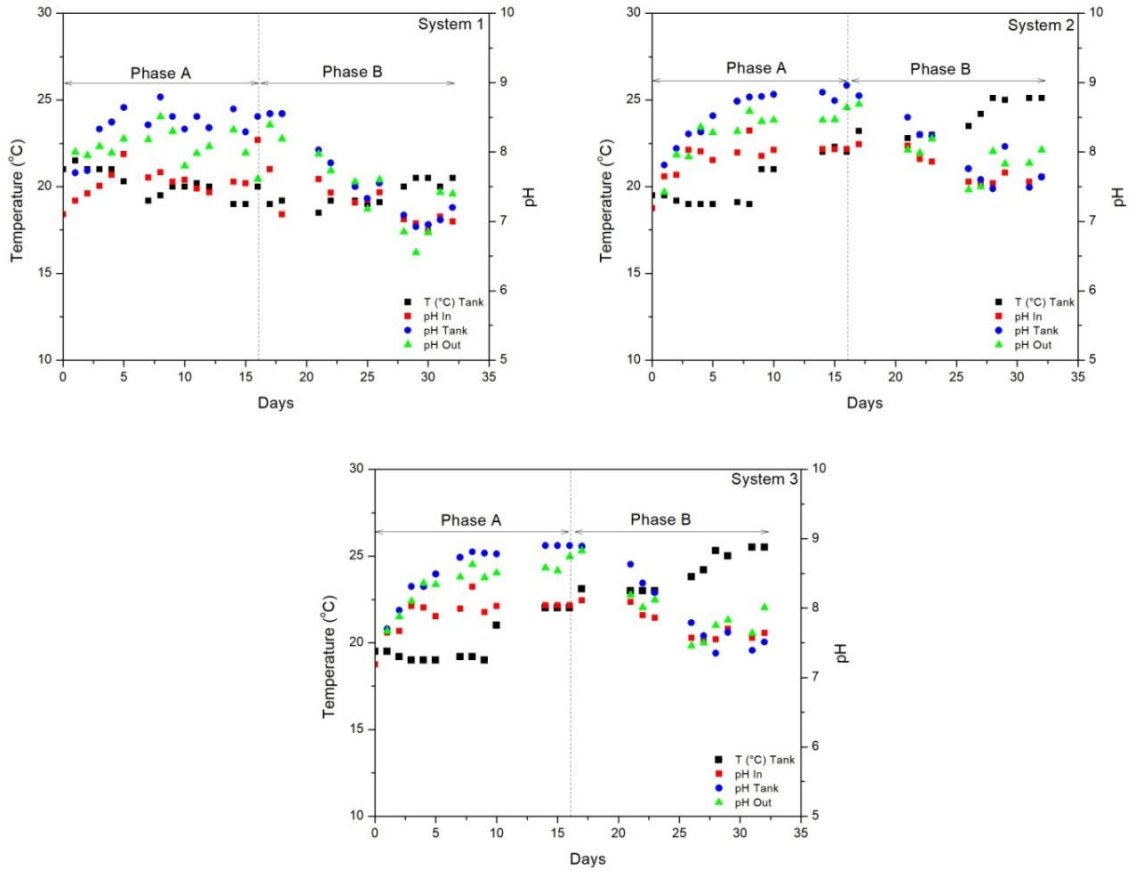


Figure 3.3.2 Daily temperature and pH variation during Phase A (0-16 Day) and B (16-32 Day) in System 1 (*L. minor*), System 2 (*L. gibba*), and System 3 (*L. minor + L. gibba*).

As it was mentioned above, daily and weekly samples were taken during Phases A and B to monitor the performance of each system. The removal of NH_4-N was significant in all systems (Figure 3.3.3a), while the highest removals were observed in the presence of *L. gibba* and in the combined presence of the two duckweeds ($p<0.05$). As a result, at Phase B (end of continuous flow experiments) the removal of NH_4-N was 90.8 ± 7.5%, 99.5 ± 0.3% and 99.5 ± 0.5% for *L.minor*, *L.gibba* and the combination of two duckweeds, respectively. The removal of TN in all systems was higher than 50% in Phase A and higher than 60% in Phase B, while no statistical difference was observed between different systems (Figure 3.3.3b). The almost total removal of NH_4-N can be explained by the fact that duckweeds prefer to use ammonium nitrogen comparing to other nitrogen forms (Wang et al., 2014 and Zhao et al., 2014).

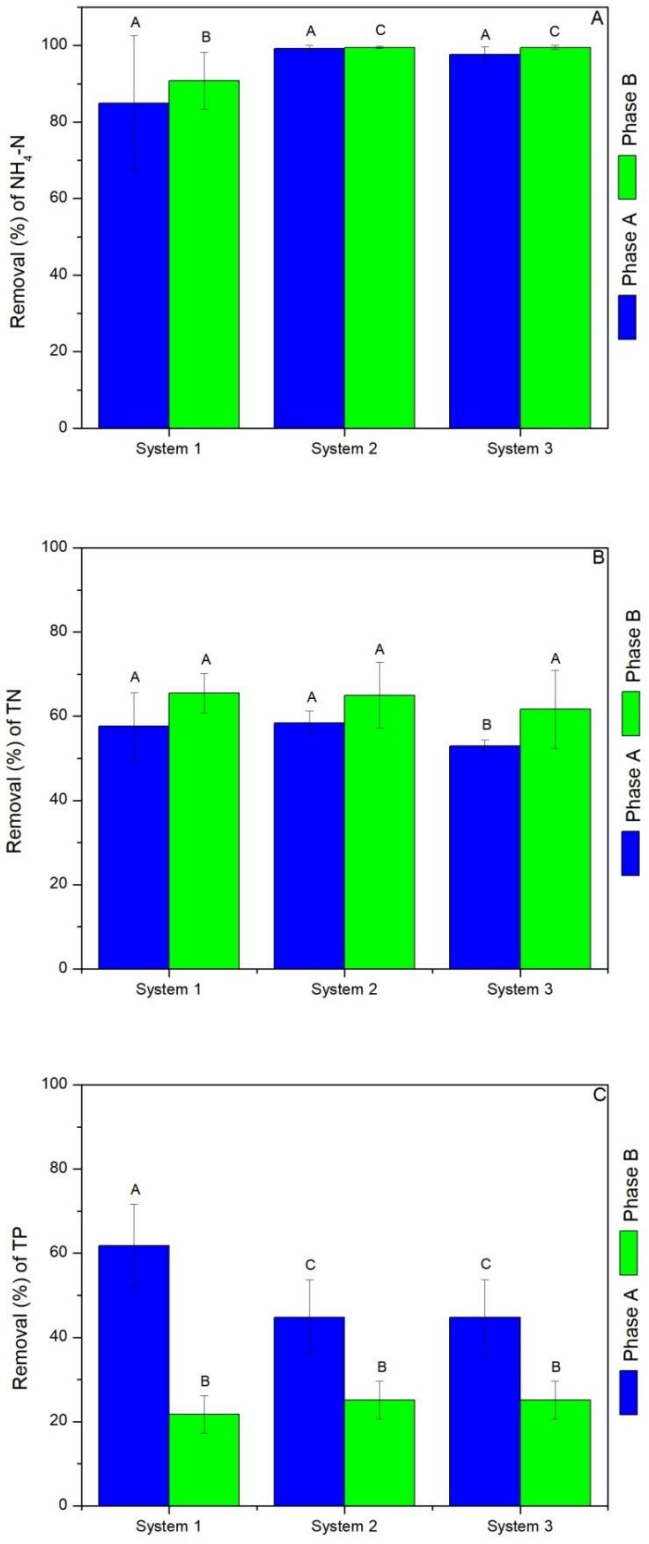


Figure 3.3.3 Removal (%) of NH_4-N (A), TN (B) and TP (C) in different experimental Systems (System 1: *L. minor*; System 2: *L. gibba*; System 3: *L. minor* + *L. gibba*) during Phase A and Phase B.

Regarding TP removal, during Phase A the highest removal was observed in System 1 with *L. minor* ranging up to 61.8 ± 9.8%. Lower TP removals were observed during Phase B, not exceeding 30% (Figure 3.3.3c). Comparable results for TP removal have also been reported in the literature for several tested media (Xu et al., 2011b, Iatrou et al., 2015, Zhao et al., 2014); however further research is needed to clarify the reasons for the decreased P removal observed in the presence of elevated NH_4-N concentrations (Phase B).

3.3.3.2 Growth of biomass, protein and starch content in different experimental systems

Determination of biomass wet weight in three systems showed that it was gradually increased during Phases A and B. This observation is consistent to the literature as according to Wang et al. (2014) the addition of 30 mg L^{-1} NH_4-N contributes to the increment of duckweeds biomass without toxic effects to the organisms.The highest biomass amount was observed in System 3 (*L. minor* + *L. gibba*) and it was 41.6 g at the end of the experiment, while the final wet weight for System 1 (*L. minor*) and System 2 (*L. gibba*) was lower reaching 30 g and 36.2 g, respectively (Figure 3.3.4a). The specific growth rates of biomass were calculated by linear regression of the natural logarithm versus culturing time according to the OECD protocol (OECD, 2006). The highest specificgrowth rate was achieved in System 3 (0.19 d^{-1}), followed by System 2 (0.17 d^{-1}) and System 1 (0.14 d^{-1}) (Figure 3.3.4b). Comparing observed growth rates in Phases A and B, it is worth mentioned that the highest growth rates were achieved in Phase B, indicating that the addition of ammonium nitrogen enhanced biomass growth (Figure 3.3.4b).

The calculation of normalized specific growth rates to the area of the tank at the end of the experiment (32 days) resulted to values equal to 8.9 g m^{-2} d^{-1} (System A), 12.1 g m^{-2} d^{-1} (System B) and 14.9 g m^{-2} d^{-1} (System C), indicating that the combination of *L. minor* and *L. gibba* achieves the highest biomass production. These values of normalized growth rates were significantly higher than those reported by Xiao et al. (2013) in experiments in the field and Zhao et al. (2014) in experiments with different duckweed species in swine wastewater.

After the measurements of the wet weight in each system and the calculation of growth rates, the biomass that had been removed was used for the determination of the crude protein and the starch content. The determination of crude protein content is crucial for a future use of produced biomass as animal feedstock, while the determination of starch content indicate the possibility of using this biomass for bioethanol production (Toyama et al., 2018).

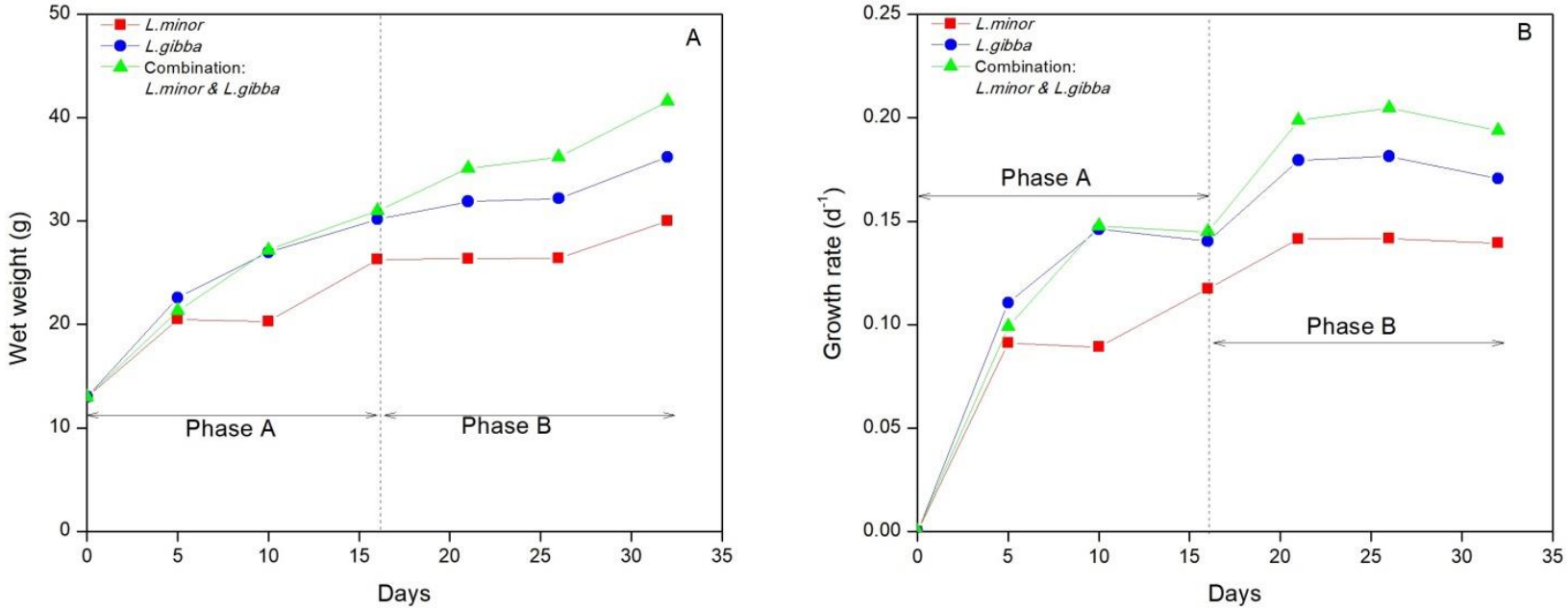


Figure 3.3.4 Wet weight (A) of biomass and growth rate (B) in System 1 (*L. minor*), System 2 (*L. gibba*) and System 3 (*L. minor* + *L. gibba*) during Phase A (0-16 Day) and Phase B (16-32 Day).

According to Table 3.3.2, at the end of Phase A the protein content ranged between 21.9% (System 1) to 25% (System 2). The addition of NH_4-N during Phase B resulted to significant increase of protein content, reaching 44.4% in System 3, 41.9% in System 2 and 39.4% in System 1. Calculation of protein productivity in Phase A (Day 16) showed that it was ranged between 3.1g m^{-2} d^{-1}(System 1) and 4.6 g m^{-2} d^{-1} (System 2); whereas it was increased significantly during Phase B, reaching 4.7 mg m^{-2} d^{-1}, 6.3 mg m^{-2} d^{-1} and 8.1 mg m^{-2} d^{-1} in System 1, 2 and 3, respectively, at Day 32.

Table 3.3.2 Crude protein content (%) in biomass originated from System 1 (*L. minor*), System 2 (*L. gibba*), System 3 (*L. minor* + *L. gibba*) during Phases A and B.

Day of experiment	System 1	System 2	System 3
	Experimental Phase A		
0	$21.3 \pm 0.2^{1,a}$	$23.8 \pm 0.2^{1,b}$	$23.1 \pm 0.2^{1,b}$
5	22.5	24.4	22.5
10	21.9	23.8	22.5
16	$21.9 \pm 0.2^{1,a}$	$25 \pm 0.2^{1,b}$	$23.1 \pm 0.2^{1,c}$
	Experimental Phase B (addition of NH_4-N)		
21	28.1	31.9	32.5
26	36.9	38.1	40
32	$39.4 \pm 0.2^{1,a}$	$41.9 \pm 0.2^{1,b}$	$44.4 \pm 0.2^{1,c}$

[1]Three replicates for crude protein at 0d, 16d and 32d. For the other days of sampling one replication;
[2]*a, b, c*: different letters indicate statistical differences at 95% confidence level between Systems 1, 2 and 3

Based on the above results, it seems that the combination of *L. minor* and *L. gibba* resulted to the highest protein content.The levels of crude protein from the current study are comparable to previous studies conducted with different media such as diluted swine, lagoon wastewater, human urine and treated wastewater where the crude protein ranged between 10% and 40% (Mohedano et al., 2012; Xu et al, 2011c; Xiao et al., 2013; Iatrou et al., 2015). In a recent study where *L. minor* was cultivated with treated wastewater, the protein content reached 25.3% in a period of 14 days

(Iatrou et al., 2015). It is worth mentioned that in the current study, the percentage of crude protein exceeded the aforementioned percentage of 25.3% in all three experiments (System 1-3). It seems that the addition of ammonium nitrogen is a crucial step for producing duckweed biomass with high percentage of crude protein.

Regarding starch content, during Phases A and B the starch content was almost stable ranging between 21.4% and 21.8% for all three experimental Systems (Figure 3.3.5). The transfer of biomass in water containing no nutrients in Day 32 resulted to a gradual increment of starch content up to the end of the experiment (Day 53). As it has been reported in the literature, the starch content of duckweed may increase after its transfer in water with absence of nutrients (Ge et al., 2012; Xiao et al., 2013). In a recent study with *L. minor* (Xu et al, 2011c), it was found that the highest starch content reached 21 days after the transfer to the water. The increase of starch content under conditions of nitrogen deficiency is due to the increased output from the gluconeogenesis and tricarboxylic acid cycle (TCA) pathways and to the decreased lipids and pectin biosynthesis (Yu et al., 2017).Statistical testing was performed for all experiments for starch accumulation of Phase C. The highest starch content was observed for the combination of the two duckweeds (46.1 ± 0.1%), followed by *L. gibba* (44.9 ± 0.1%) and *L. minor* (43.9 ± 0.1%). These differences were statistically significant ($p<0.01$), indicating the highest starch content of the combination of two duckweeds.The results of the current study are comparable with the results of Iatrou et al. (2015), where in *L. minor* cultures the starch content reached 45.9% in absence of nutrients within 21 days.Similar results were obtained when starch productivity was calculated. Specifically, starch productivity in Phase A and B was low in all Systems, not exceeding3.2 g m^{-2} d^{-1}(System 3, Day 32). On the other hand, significant increasewas observed in Phase C where values equal to 5.6g m^{-2} d^{-1}, 6.7g m^{-2} d^{-1} and 8.1g m^{-2} d^{-1} were calculated for System 1, 2 and 3, respectively, at the end of the experiment.

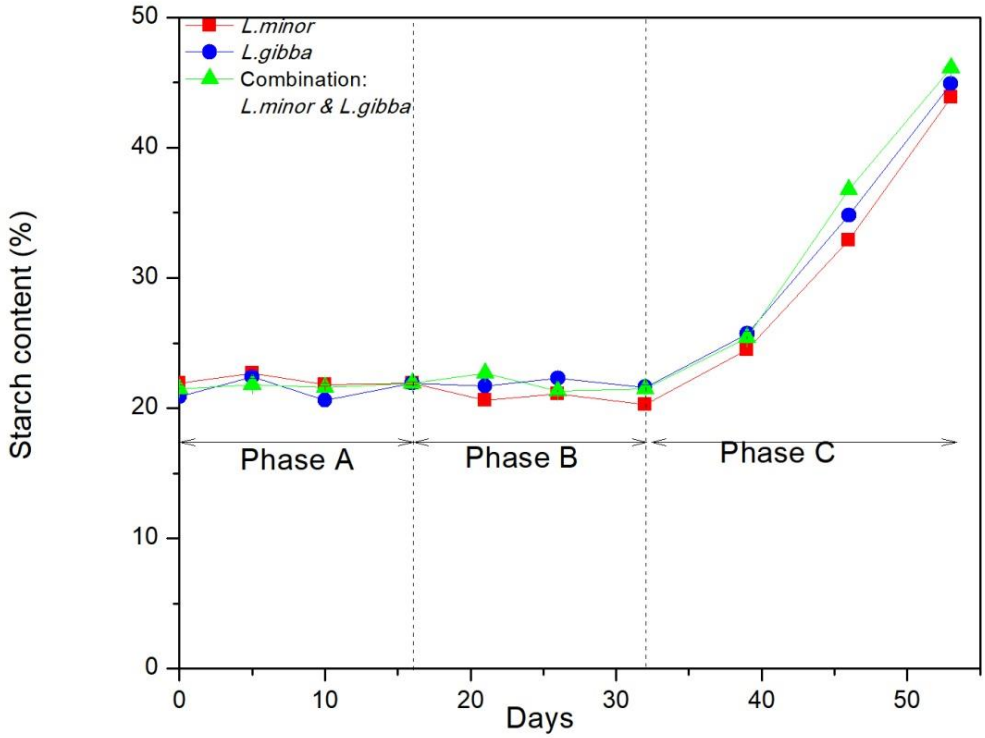


Figure3.3.5 Starch content (%) in biomass originated from System 1 (*L. minor*), System 2 (*L. gibba*) and System 3 (*L. minor* + *L. gibba*) during Phases A, B and C.

According to the current study a duckweed-based system seems to be an effective alternative method compared to conventional wastewater treatment systems. Some of the advantages of such a system are the minimum required operational and maintenance costs, the high quality of produced water and the potential reuse of the biomass for several used. On the other hand, further improvements are still needed in order to reduce the required area for their construction. Future parallel studies should be conducted to compare the performance and cost of such systems with other types of constructed wetlands commonly used for wastewater treatment and biomass valorisation (Tsihrintzis et.al, 2007; Yang et.al, 2015).

3.4 Removal of antimicrobials in batch and continuous flow reactors planted with *Lemna minor* (Paper IV)

3.4.1 Introduction

Antimicrobials have been extensively used for humans and animals against microbial infections. After their consumption, they are metabolized and they are excreted through urine and faeces into sewage either as unchanged parent molecules or as metabolites. According to previous studies, conventional primary and secondary wastewater treatment can only partially remove these compounds (Matamoros et al., 2012; Verlicchi et al., 2012; Zhang et al., 2014; Ahmed et al., 2015). As a result, tertiary treatment technologies are needed to achieve full elimination from wastewater and decrease the risk for the environment due to wastewater discharge (Thomaidi et al., 2015).

During the last decades, constructed wetland technology has been widely used for tertiary wastewater treatment. Among different plant-based systems, ponds with duckweed (*L. minor*) have been applied with success in different countries for the removal of nutrients and organic matter (Dosnon – Olette et al., 2010; Reinhold et al., 2010; Haarstad et al., 2012), combining efficient wastewater treatment and important biomass production. This organism is a floating freshwater aquatic plant commonly found in lakes and streams that has been used for phytoremediation purposes, due to its tension to uptake heavy metals from water and wastewater (Sekomo et al., 2012; Pietrini et al., 2016; Rezania et al., 2016).

Despite the extended use of *L. minor* systems, so far, there are few research papers investigating the elimination of organic micropollutants in such systems (Reinhold et al., 2010; Matamoros et al., 2012; Iatrou et al., 2015). Matamoros et al. (2012) studied the removal of seven micro contaminants in a reactor with standard growth solution planted with *L. minor*. Their findings revealed that diclofenac, triclosan and caffeine were totally removed, following by ibuprofen and naproxen with a removal higher than 80% and 60%, respectively. In another research, Reinhold et al. (2010) noticed that duckweed actively increased the removal of some pharmaceuticals, personal care products (PCPs) and pesticides. In another study, Iatrou et al. (2015) showed the simultaneous biomass production, removal of nutrients and elimination of two

antimicrobials (ciprofloxacin and sulfamethoxazole) in *L. minor* experiments with human urine and domestic wastewater. In most of the aforementioned studies, the removal of micropollutants in *L.minor*systems has been faced as a black box and the contribution of different mechanisms on micropollutants removal has not been evaluated. On the other hand, it is widely known that some of the organic micropollutants are subjected to hydrolysis, photodegradation and reductive transformation (Zhang et al., 2014; Zeng et al., 2012), while sorption to biomass and plant uptake are important mechanisms for the removal of toxic compounds in duckweed systems (Zhang et al., 2014; Pietrini et al., 2016; Rezania eta al., 2016). Moreover, the transformation products (TPs) of these compounds in the absence and presence of *L. minor* have not been identified.

Based on the above, the main objectives of this study were to investigate the removal of four antimicrobials, cefadroxil (CFD), metronidazole (METRO), trimethoprim (TMP) and sulfamethoxazole (SMX) (Table S3.4.1), from water and treated wastewater using *L. minor* bioreactors and to identify plant and not plant-associated processes responsible for their elimination. The possible toxicity of these compounds in *L. minor* was initially checked in single and mixture toxicity experiments. Afterwards, batch experiments were carried out to study the role of photodegradation, hydrolysis, sorption and plant uptake on target compounds removal. The kinetics constants of target compounds were calculated; while their TPs were identified using LC-QTOF-MS technique. Finally, a continuous flow lab-scale system planted with fresh duckweed was used to investigate the removal of two target compounds (METRO and TMP) from secondary treated wastewater in different ponds. These micropollutants showed the highest and the lowest affinity for plant uptake in batch experiments. A mass balance model was applied to describe the contribution of different mechanisms to target compounds removal. Biodegradation was also included in this model using biodegradation rate constants found from the literature.

3.4.2 Materials and Methods

3.4.2.1 Chemicals and Reagents

Analytical standards of CFD, METRO, TMP and SMX were purchased from Sigma – Aldrich Fluka (Steinheim, Germany). Stock solutions were prepared in pure water for batch experiments and in methanol (MeOH) for the continuous-flow experiments. HPLC grade water was prepared in the laboratory using a MilliQ/MilliRO Millipore system (Bedford, USA), while MeOH (LC-MS grade) was obtained from Fisher (USA). Strata – X polymeric reversed phase SPE cartridges (200mg/6ml) and RC filters (0.2μm, 4mm) were purchased from Phenomenex (Torrance, CA, USA). Duckweed communities were donated from Federal Environment Agency (Berlin, Germany).

3.4.2.2 Toxicity experiments

The duckweed *L. minor* cultures were initially grown for 4 weeks in Swedish standard sterile growth medium (Medium SIS) in accordance with the conditions described by OECD Guideline 221 (OECD, 2006). Toxicity range finding tests were conducted to check the possible effects of the target compounds on *L. minor*, individually as well as in mixture (OECD, 2006). The concentrations, which were tested for CFD and METRO were 10, 100, 1000 and 10000 μg L^{-1}, while the tested concentrations for TMP and SMX were 2, 20, 200 and 2000 μg L^{-1}. For mixture toxicity, three different levels were tested; 150, 200 and 250 μg L^{-1}. All toxicity experiments were performed in triplicates in glass petri dishes, containing 100 mL Medium SIS with 12 healthy fronds of *L. minor* per petri dish. Stock cultures and cultures of toxicity experiments were incubated in a temperature - controlled chamber at 24 ± 0.5˚C under continuous illumination with fluorescent lamps (OSRAM, FQ 39W/840 HO). The duration of each experiment was 7 days and the estimation of inhibition was based on the frond number calculation of specific growth rate, according to Gatidou et al. (2015).

3.4.2.3 Batch experiments for removal mechanisms investigation

Four different batch reactor systems were used to investigate the aqueous removal of target compounds due to hydrolysis, photodegradation, sorption and plant uptake (Table S3.4.2, Experiments A to D). All experiments were performed in triplicate, in glass flasks that contained 100 mL SIS sterile growth medium according to the conditions described by OECD Guideline 221 (OECD, 2006). Flasks were placed in

incubator chambers under constant light for a period of 24 d. The temperature was 24.0 ± 0.5˚C, pH was 7.0 ± 0.2 and the initial concentration of target compounds was 250 μg L^{-1}. Samples were taken at different time intervals (0, 6, 24, 48, 72, 120, 168, 216, 264, 336, 408, 504 and 576 h).

Experiment A was conducted in the absence of *L. minor* under dark conditions to investigate the hydrolysis of antimicrobials. Experiment B was conducted under light conditions and both hydrolysis and photodegradation accounted for the elimination of target compounds. To investigate the contribution of sorption on target organism, *L. minor* communities were exposed in 1 g L^{-1} sodium azide for 7 d and rinsed with Medium SIS prior the addition to experimental reactors (Experiment C) (Tront and Saunders, 2006; Reinhold et al., 2010). For the investigation of antimicrobials' uptake by *L. minor,* 2 gr of fresh organism were added in each flask (Experiment D). This plant density mimicked full surface coverage growth observed in natural and treatment wetlands (Tront and Saunders, 2006; Reinhold et al., 2010). It is worth mentioned that all four studied mechanisms is expected to contribute to the removal of target compounds in this set of experiments.

3.4.2.4 Batch experiments for transformation products identification

Additional batch experiments were conducted to study the TPs of target antimicrobials in the presence and absence of *L. minor* (Table S3.4.3). All experiments were performed in triplicate, in glass flasks that contained 100 mL Medium SIS. Flasks were placed in the same incubator chambers under constant light for a period of 24 days. The temperature was 24.0 ± 0.5˚C, pH was 7.0 ± 0.2 and the initial concentration of target compounds was 1000 μg L^{-1} (Table S3.4.3). Target compounds were added individually in each flask, while water samples were taken at the 7^{th} and 24^{th} day of the experiment.

3.4.2.5 Continuous - flow system: set up and operation

The continuous flow set-up comprised from one treatment line with three duckweed mini ponds in series (Figure S3.4.1). Each pond had a working volume of 5 L and duckweed biomass was harvested every week in order to maintain a density of 600 g

fresh weight per m^2 (Sekomo et al., 2012). The system operated with a hydraulic retention time (HRT) of 6.5 days per tank, under 16/8h light/darkness, respectively. Evapotranspiration losses were counterbalanced daily by adding tap water.

The fed up of duckweed system was conducted using secondary biologically treated wastewater, originating from University Campus Sewage Treatment Plant (STP). This STP consists of a nitrifying activated sludge bioreactor and a secondary clarifier. After an initial start-up period of 3 months to stabilize flow rate and to allow duckweed acclimatization and growth onto wastewater, wastewater was spiked with target antimicrobials in order to achieve a concentration of around 10 μg L^{-1} in the inlet of the lab-scale system. System was operated under these conditions for a period of 79 days. During this phase, its performance was monitored for conventional pollutants as well as for target antimicrobials (METRO and TRI). The sampling for the determination of antimicrobials was started 22 days after spiking with target compounds (time equal to system's total HRT). Wastewater samples were taken from four sampling points as indicated in Figure S3.4.1.

3.4.2.6 Analytical methods

Analysis of convention pollutants

To control the operation of continuous-flow system, chemical oxygen demand (COD), biological oxygen demand (BOD), ammonia-N (NH_4-N), nitrate-N (NO_3-N), total phosphorous (TP) and total suspended solids (TSS) were determined in a regular basis at Points A to D (Figure S3.4.1), according to Standard Methods (APHA-AWWA-WPCF, 2005). DO, temperature, pH and conductivity were also measured in a daily basis, using portable instruments.

Analysis of target antimicrobials

For the determination of target compounds in batch experiments, aqueous samples (0.6 ml) were taken regularly during the experiment, filtered through 0.2 μm Whatman PTFE filters, mixed with MeOH and analyzed by a Shimatzu (Japan) LC20-AD prominence liquid chromatographer associated with a SPD-M20A prominence diode array detector and a SIL-20AC auto sampler. The analytical procedure was based on a previously published method (Ašperger et al., 2009). Antimicrobials were

separated from medium components using isocratic separation with aqueous 0.5% HCOOH in 0.05M CH_3COONH_4:MeOH (70:30, v/v) at a flow rate of 0.7 ml min^{-1}. Chromatographic separation was achieved with a Zorbax reverse phase SB–C18 analytical column (150 x 4.6 mm; 5 μm, Agilent) at 30 ˚C, using a regard column SB-C18. The acquisition wavelengths were 280 nm for METRO and 270 nm for CFD, TMP and SMX. The method limit of detection for each antimicrobial was 2.0 μg L^{-1} for CFD, 0.4 μg L^{-1} for METRO, 2.5 μg L^{-1} for TRI and 0.4 μg L^{-1} for SMX.

Solid phase extraction (SPE) was used for the determination of antimicrobials in wastewater samples originating from continuous-flow experiments (Dasenaki and Thomaidis, 2015). Samples pH was initially adjusted to 2.5 by using HCL (0.1M) and from each sample 100 ml was used with an addition of 1 ml EDTA 0.1% (w/v). The C18 cartridges were conditioned by 3 x 2 ml MeOH and 3 x 2 ml pure H_2O. After the samples were passed through cartridges with a normal flow rate, the cartridges were washed with 2 ml pure H_2O and then vacuum dried for 60 min. The compounds were eluted with 2 x 3 ml MeOH, the eluates were evaporated to dryness under a steam of nitrogen (1 bar) at 35 ˚C and re-dissolved in 0.5 ml of the initial mobile phase (25% MeOH – 75% H_2O with HCOOH 0.05%). After filtration through the syringe filters (0.2 μm RC filters, Phenomenex), they were analyzed through a liquid chromatography–tandem mass spectrometry (LC-MS/MS) system. Chromatographic separation was performed with an Atlantis T3 C18 column (100 mm×2.1 mm, 3 μm) with a gradient elution using for mobile phase water containing 0.01% (v/v) formic acid and methanol in positive ionization mode. The method limit of quantification for each antimicrobial was 7.4 ng L^{-1} for METRO and 5.2 ng L^{-1} for TRI, while their recoveries were ranged from 102% (METRO) to 107% (TRI).

Identification of transformation by-products

An ultrahigh-performance liquid chromatography (UHPLC) system (DionexUltiMate 3000 RSLC, Thermo Fisher Scientific) coupled to a quadrupole-time-of-flight mass spectrometer (QTOF-MS) (Maxis Impact, Bruker Daltonics) was used for the screening analysis and the identification of candidate TPs. For the chromatographic separation, a Thermo Dionex Acclaim RSLC C18 column (2.2 μm, 120 Å, 2.1×100 mm), thermostated at 30 oC, was used. The QTOF spectrometer was equipped with

electrospray ionization interface operating in positive ionization mode, with the following operation parameters: capillary voltage, 2500 V; end plate offset, 500 V; nebulizer pressure, 2 bar (N2); drying gas, 8 L min−1 (N2); drying temperature, 200 °C.

For the detection and identification of tentative TPs, suspect and non-target screening workflows were applied. Regarding suspect screening, samples were screened by extracting the exact masses of the potential TPs, according to a suspect database established for each compound. To accomplish that, in-silico metabolite/transformation/degradation prediction tools, such as the online pathway prediction system hosted by EAWAG institute (EAWAG-PPS) and MetabolitePredict software (Bruker Daltonik), were applied. In non-target screening, the initial crucial step is subtraction of each control sample from its respective treated sample, to expose masses that are exclusively detected in the treated samples. This was achieved using Bruker Compass MetaboliteDetect 2.0 software, which allows the sophisticated comparison of two full scan LC-MS data sets, creating a peak list containing exact mass and retention time (Rt) information. SmartFormula algorithm was then used to create possible sum formulaes for each exact mass, taking into account element restrictions (C, H, N, O and S), mass tolerance 5 mDa, the hydrogen to carbon ratio (H/C) from 0 to 3, check for ring and double bonds and electron configuration even for the MS and both odd and even for MS/MS peak. The analytical evidence supporting each tentative TP was variable and as a result different identification confidence levels were assigned (Schymanski et al., 2014). Detailed information for the chromatographic separation and the methodology applied for TPs identification can be found in Supplementary Material (Transformation products identification).

Calculations

The obtained data from batch experiments A to D (Table S3.4.2) were described by the pseudo first-order kinetics, (Equation 3.4.1):

$$C_t = C_o e^{-k_i t} \quad (3.4.1)$$

Where C_t and C_0 are the target compound concentrations in batch experiment at time t and $t = 0$, respectively, (μg L^{-1}), k_i is the removal rate constant for each experiment (d^{-1}), and *i* the relevant experiment (A, B, C or D).

The calculation of hydrolysis rate constant ($k_{hydrolysis}$), photodegradation rate constant ($k_{photodegradation}$), sorption rate constant ($k_{sorption}$) and plant uptake rate constant (k_{uptake}) was conducted using the following Equations (3.4.2-3.4.5) (OECD, 2008):

$$k_{hydrolysis} = k_A \quad (3.4.2)$$

$$k_{photo\ deg\ radation} = k_B - k_A \quad (3.4.3)$$

$$k_{sorption} = k_C - k_B \quad (3.4.4)$$

$$k_{uptake} = k_D - k_C \quad (3.4.5)$$

Where k_A, k_B, k_C and k_D the removal rate constants for the experiments A, B, C and D, respectively.

Having in mind that the target compounds are not subjected to volatilization, Equations 3.4.6 and 3.4.7 were used to predict their fate in *L. minor* continuous-flow system:

$$M_{in} = M_{hydrolysis} + M_{photo\ deg\ radation} + M_{sorption} + M_{uptake} + M_{bio\ deg\ radation} \quad (3.4.6)$$

Where M_{in} and M_{out} are the masses of investigated compounds in influents and effluents, respectively (μg d^{-1}), $M_{hydrolysis}$, $M_{photodegradation}$,,$M_{sorption}$,M_{uptake} and $M_{biodegradation}$ are the masses of investigated compounds that are hydrolysed, photodegraded, sorbed, uptaked by *L. minor* and biodegraded, respectively (μg d^{-1}).

$$C_{in} Q_{in} = (k_{hydrolysis}\ C_{out} V) + (k_{photo\ deg\ radation}\ C_{out} V) + (k_{sorption}\ C_{out} V) + \\ + (k_{uptake}\ C_{out} V) + (k_{bio\ deg\ radation}\ C_{out} V) + (Q_{out} C_{out}) \quad (3.4.7)$$

Where C_{in} and C_{out} are the concentrations of investigated compounds in influents and effluents, respectively (μg L^{-1}), Q_{in} and Q_{out} are the flow rates in influents and effluents, respectively (L d^{-1}), V is the total volume of the system (L) and $k_{biodegradation}$ is the biodegradation rate constant for each target compound found from the literature (d^{-1}).

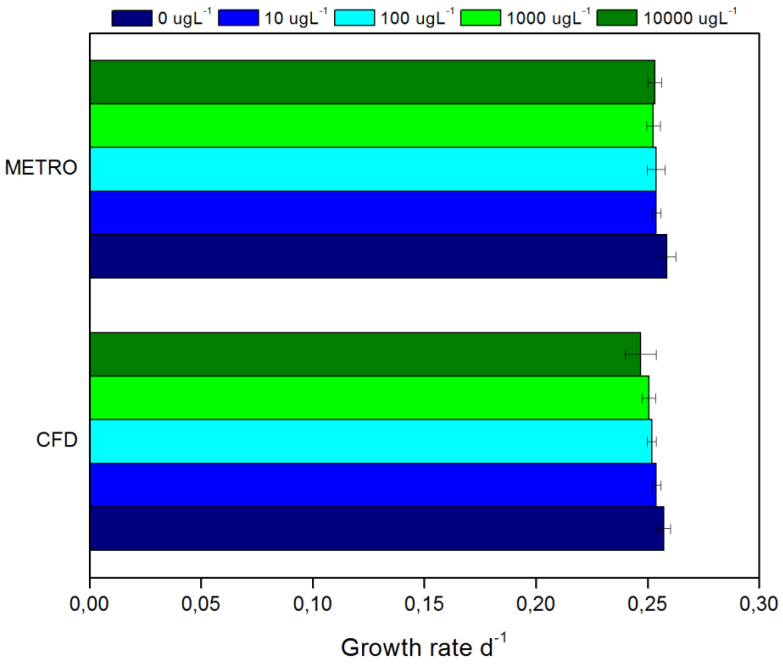
OriginPro 8 SR0 (Version 8.0724, OriginLab Corporation) was used for the construction of all graphs in current study. The toxicity range finding tests were checked with SPSS 17.0 by one-way ANOVA.

3.4.3 Results and Discussion

3.4.3.1 Toxicity experiments

The toxicity of target compounds on *L. minor* was tested in single and joint toxicity experiments. According to the results of single toxicity experiments, in all experiments, leafs were green with no chlorosis effects, while no statistical differences ($p > 0.05$) were observed on specific growth rates of *L. minor* for concentrations of CFD and METRO up to 10000 μg L^{-1} as well as for concentrations of TMP and SMX up to 2000 μg L^{-1} (Figure 3.4.1a).

So far, there is limited information available in the literature for the toxicity of target antimicrobials on *L. minor*. Kołodziejska et al. (2013) reported no toxicity of METRO for concentration up to 25000 μg L^{-1}, while in a recent study, different SMX concentrations were tested and no toxic effects observed for concentrations up to 2000 μg L^{-1} (Iatrou et al., 2015). To the best of our knowledge, no data is available for the effect of CFD and TRI on studied organism. To investigate the possible joint toxicity of target compounds, specific growth rates were also calculated for different mixtures of antimicrobials and compared with the control culture. No statistically ($p > 0.05$) significant decrease of growth rates values were observed for concentrations of 150, 200 and 250 μg L^{-1} (Figure 3.4.1b). Based on the above, the concentration of 250 μg L^{-1} for each target compound was selected for the elaboration of batch experiments reported in Paragraph 3.4.3.2.



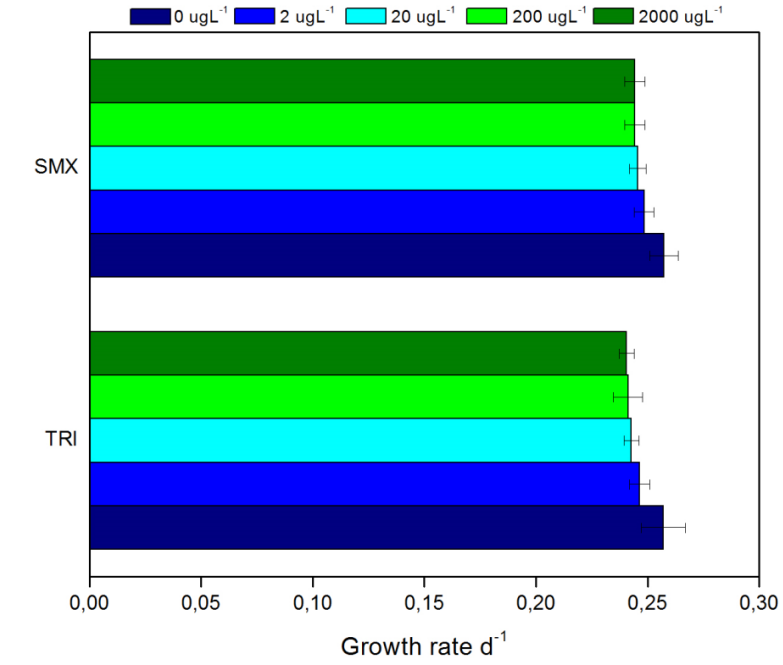


Figure 3.4.1a Calculation of specific growth rates of *L. minor* in experiments conducted in the absence and presence of target antimicrobials, results from single toxicity experiments.

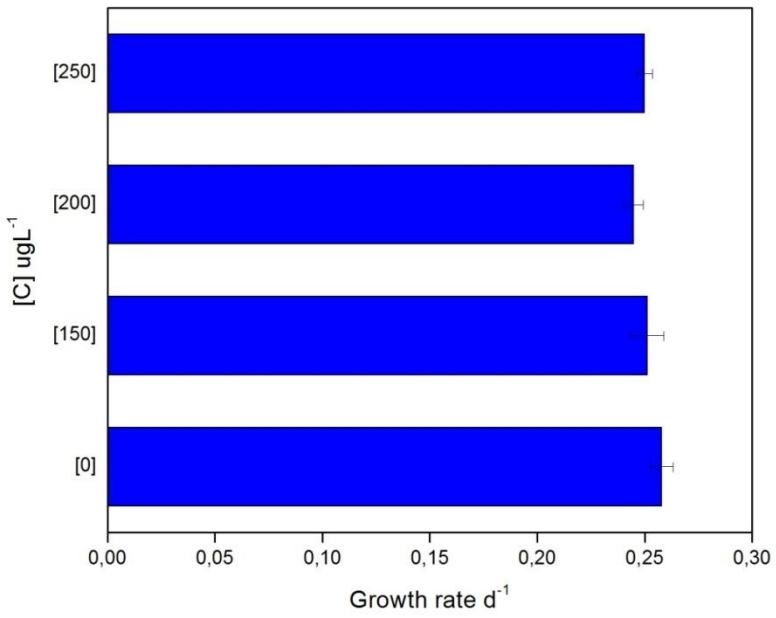


Figure 3.4.1b Calculation of specific growth rates of *L. minor* in experiments conducted in the absence and presence of target antimicrobials, results from joint toxicity experiments.

3.4.3.2 Fate of target antimicrobials in batch experiments

The removal of target antimicrobials in batch experiments conducted under different experimental conditions is shown in Figure 3.4.2 and Table S3.4.4. According to the results of Experiment A, hydrolysis contributed significantly to the removal of CFD, as its concentration decreased by more than 70% up to the end of the experiment (24 d), under dark conditions and absence of biomass. On the other hand, the role of hydrolysis was of minor importance for the other compounds, resulting to removal of 20 ± 2 for TRI, 12 ± 1 for SMX and 11 ± 2 for METRO (Figure 3.4.2 A). The presence of light in Experiment B enhanced slightly the removal of all target compounds, while the highest removal efficiency was observed for CFD and it was equal to 92 ± 0 (Figure 3.4.2 B).

Experiments with inactivated biomass (Experiment C) showed that the mechanism of sorption increased to some extent the removal of METRO, TRI and SMX, while it accelerated significantly CFD removal, as full elimination for this compound was observed 336 h (14 d) after the start of the experiment (Figure 3.4.2 C). Finally, the

use of fresh *L. minor* in batch Experiments D enhanced significantly the removal of all target antimicrobials (Figure 3.4.2 D, Table S3.4.4), indicating the significant role of plant uptake on their removal. Specifically, removal equal to 96 ± 0, 73 ± 0 and 59 ± 1 was observed for METRO, SMX, and TRI, respectively up to the end of the experiment. Plant uptake seems also to be an important mechanism for CFD removal, as in these experiments full elimination was observed 264 h (11 d) after the start of the experiment. It is known that the uptake and translocation of organic micropollutants within plants are driven by diffusion. After being taken up into plant tissues, these compounds might be degraded via the metabolic processes (phytodegradation). The possible biochemical reactions include transformation of parent compounds, conjugation of metabolites with macromolecules and incorporation of conjugated products into plant cell walls and vacuoles (Zhang et al., 2014; Li et al., 2014).

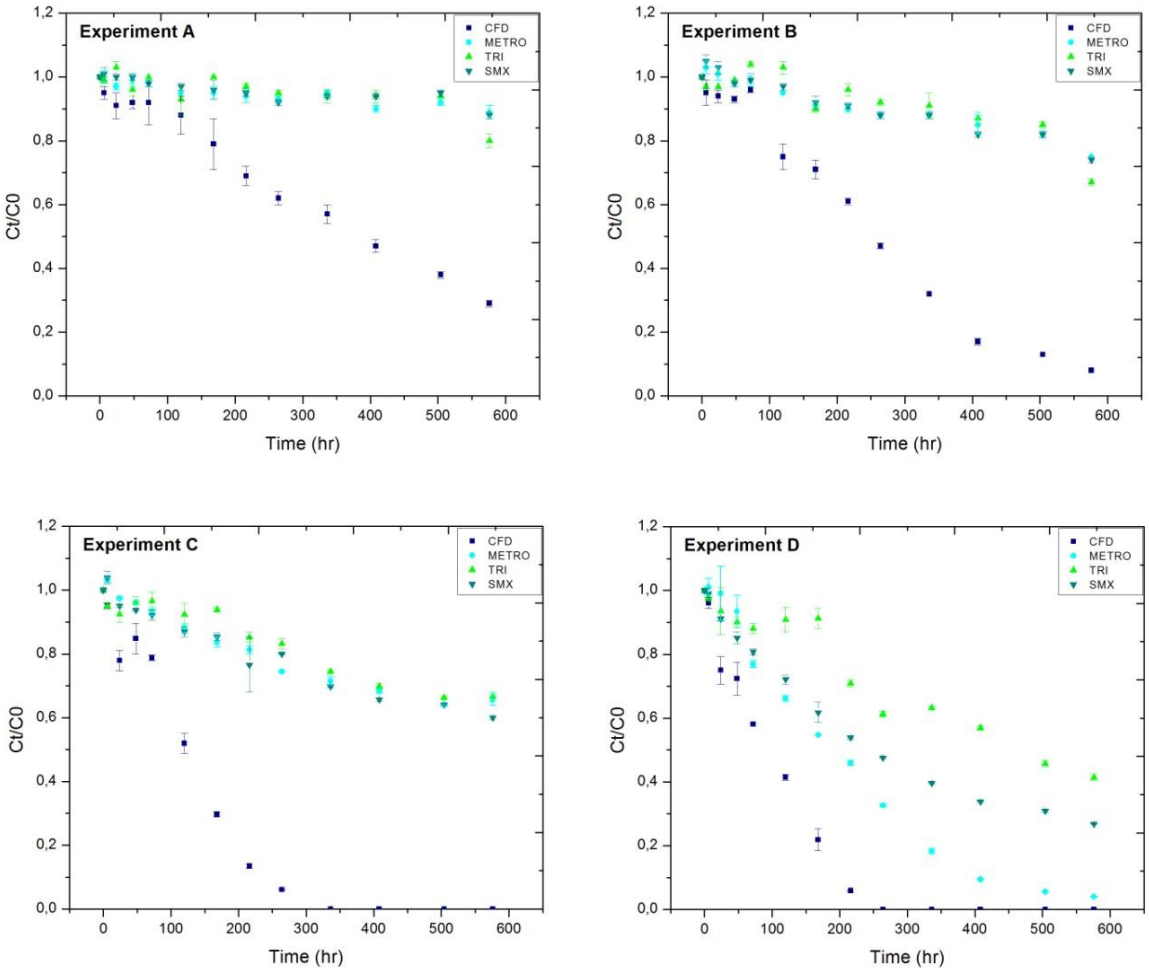


Figure 3.4.2 Elimination of tested antimicrobials in batch experiments conducted in absence of *L. minor* and dark conditions (Experiment A), in absence of *L. minor* and light conditions (Experiment B), in presence of inactivated *L. minor* and light conditions (Experiment C) and in presence of fresh active *L. minor* and light conditions (Experiment D).

The rate constants obtained in Experiments A to D and the relevant half-life values are reported in Table 3.4.1. In Experiment D that included all studied mechanisms (hydrolysis, photodegradation, sorption and plant uptake), the lower half-life value was calculated for CFD (2.5 ± 0.1 d), while the higher for TRI (20 ± 0.8 d). To the best of our knowledge, this is the first study investigating the removal and the half-lives of target antimicrobials in *L. minor* systems. In experiments with mesocosms planted with macrophytes, Cardinal et al. (2014, 2016) reported half-life values for SMX equal to 7.6 and 17 d, respectively, which is close to the value obtained in this study (12 d). In another study, Møller et al. (2016) studied the removal SMX, METRO and TRI in a wastewater stabilization pond and reported half-lives for combined hydrolysis and photodegradation equal to 118 d, 11 d and 61 d, respectively. In Experiment B of the current study, the relevant half-life values for SMX, METRO and TRI were 55 ± 1, 62 ± 1 and 65 ± 3.3 d, respectively.

Table 3.4.1 First order kinetics (k), half-life ($t_{1/2}$) and correlation coefficients (R^2) values calculated in batch experiments conducted under different experimental conditions.

		CFD	**METRO**	**TRI**	**SMX**
Experiment A	**k (d^{-1})**	0.049 ± 0.001	0.005 ± 0.000	0.006 ± 0.000	0.004 ± 0.000
	R^2	0.971	0.865	0.607	0.762
	$t_{1/2}$ (d)	14.3 ± 0.3	151 ± 12	127 ± 10	165 ± 15
Experiment B	**k (d^{-1})**	0.104 ± 0.001	0.011 ± 0.000	0.011 ± 0.001	0.013 ± 0.000
	R^2	0.960	0.957	0.716	0.954
	$t_{1/2}$ (d)	6.6 ± 0.0	62 ± 1	65 ± 3.3	55 ± 1

Experiment C	**k (d^{-1})**	0.243 ± 0.004	0.020 ± 0.000	0.018 ± 0.001	0.022 ± 0.000
	R^2	0.938	0.951	0.933	0.948
	$t_{1/2}$(d)	2.9 ± 0.0	34 ± 1	39 ± 2.1	32 ± 0.2
Experiment D	**k (d^{-1})**	0.279 ± 0.008	0.140 ± 0.002	0.036 ± 0.001	0.057 ± 0.000
	R^2	0.918	0.981	0.984	0.983
	$t_{1/2}$ (d)	2.5 ± 0.1	4.9 ± 0.1	20 ± 0.8	12 ± 0.0

To clarify the role of each mechanism on target compounds removal, kinetic constants of individual mechanisms were calculated as described in Paragraph 3.4.2.6 (Equations 3.4.2 to 3.4.5) and the results are presented in Table 3.4.2. Significant differences were observed on the values of kinetic constants according to the target antimicrobial and the studied mechanism. For three of the four studied substances (METRO, TRI, SMX), the kinetic constants of plant uptake were by far higher comparing to those of the other mechanisms, while the higher kinetic constant of CFD was observed for the sorption to biomass. Among the four compounds, the highest photodegradation and hydrolysis kinetic constants were calculated for CFD (0.055 ± 0.001 and 0.049 ± 0.001, respectively), while the relevant values for the other compounds were pretty lower, not exceeding 0.009 ± 0.000 d (SMX). To the best of our knowledge, no data is available in the literature for the hydrolysis and photodegradation constants of CFD and METRO. In previous studies, it has been reported that hydrolysis is of minor importance for SMX and TRI, an observation that is consistent to our results (Lam et al., 2004; Yu et al., 2011; Garcia-Rodriguez et al., 2013).

Table 3.4.2 Calculated values of hydrolysis rate constant ($k_{hydrolysis}$), photodegradation rate constant ($k_{photodegradation}$), sorption rate constant ($k_{sorption}$) and plant uptake rate constant (k_{uptake}) in batch experiments conducted with different antimicrobial compounds.

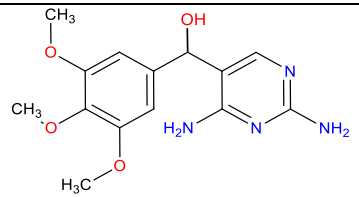
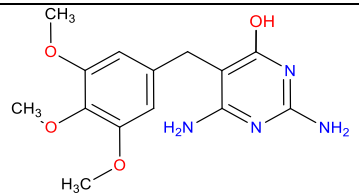
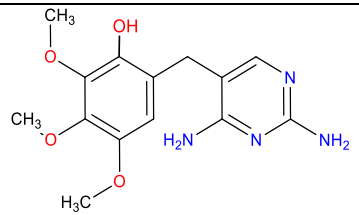
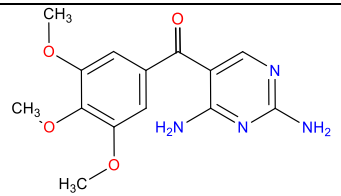
	$k_{hydrolysis}$ **(d^{-1})**	$k_{photodegradation}$ **(d^{-1})**	$k_{sorption}$ **(d^{-1})**	k_{uptake} **(d^{-1})**
CFD	0.049 ± 0.001	0.055 ± 0.001	0.139 ± 0.004	0.036 ± 0.009

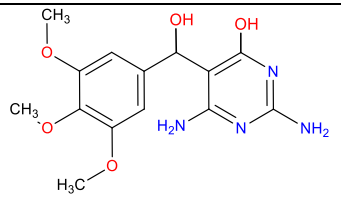
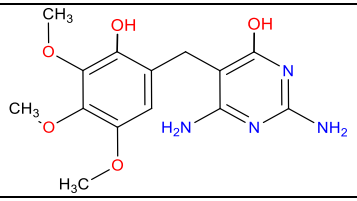
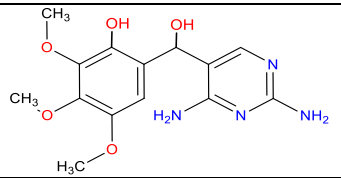
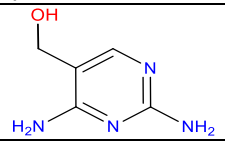
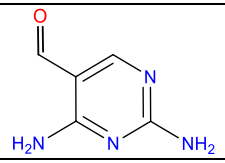
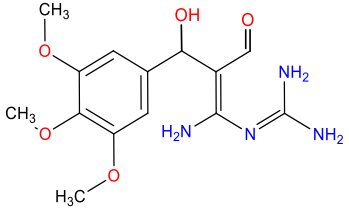
METRO	0.005 ± 0.000	0.006 ± 0.000	0.009 ± 0.000	0.120 ± 0.002
TRI	0.006 ± 0.000	0.005 ± 0.001	0.007 ± 0.001	0.018 ± 0.001
SMX	0.004 ± 0.000	0.009 ± 0.000	0.009 ± 0.000	0.035 ± 0.000

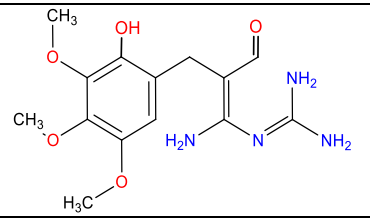
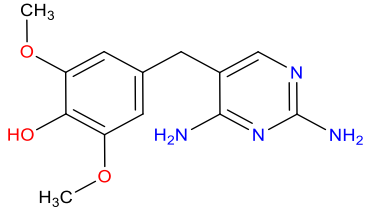
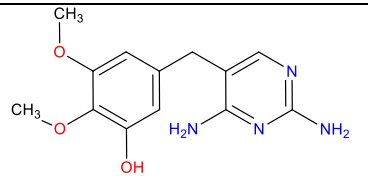
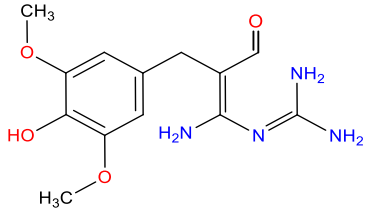
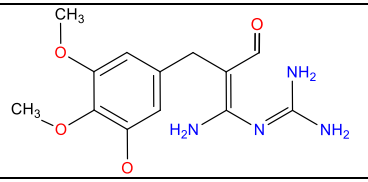
3.4.3.3 Identification of transformation products

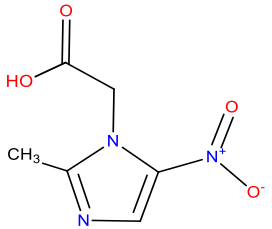
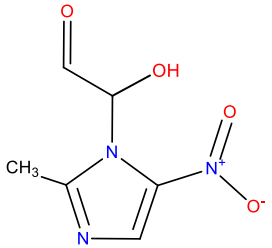
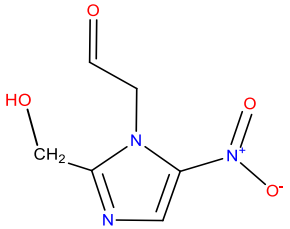
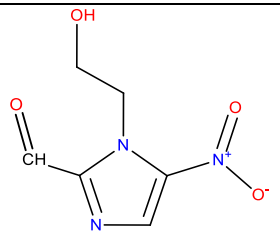
As it has been mentioned in Paragraph 3.4.2.4, additional batch experiments were conducted in absence and presence of *L. minor* and higher initial concentrations of target antimicrobials (1000 μg L^{-1}) in order to identify the TPs and degradation pathways of target antimicrobials. Table 3.4.3 summarizes all the TPs detected during the experiments, containing data regarding retention time, exact mass, proposed molecular formula, proposed structure and identification confidence level for each TP.

Table 3.4.3 Description of candidate TPs observed in batch experiments.

Parent compound	TP	m/z	Rt (min)	Molecular Formula	Tentative Structures	Id. Conf. Level
Trimethoprim	Trimeth-306a	306.1328	4.9	$C_{14}H_{18}N_4O_4$		2b
	Trimeth-306b	306.1328	4.3	$C_{14}H_{18}N_4O_4$		3
	Trimeth-306c	306.1328	5.5	$C_{14}H_{18}N_4O_4$		3
	Trimeth-304	304.1172	6.7	$C_{14}H_{16}N_4O_4$		2b

	Trimeth-322a	322.1277	5.3	$C_{14}H_{18}N_4O_5$		3
Trimethoprim	Trimeth-322b	322.1277	5.5	$C_{14}H_{18}N_4O_5$		3
	Trimeth-322c	322.1277	5.9	$C_{14}H_{18}N_4O_5$		3
	Trimeth-141	140.0698	1.5	$C_5H_8N_4O$		2b
	Trimeth-139	138.0542	1.3	$C_5H_8N_4O$		2b
	Trimeth-324a	324.1434	5.3	$C_{14}H_{20}N_4O_5$		3

	Trimeth-324b	324.1434	5.5	$C_{14}H_{20}N_4O_5$		3
Trimethoprim	Trimeth-276a	276.1222	5.1	$C_{13}H_{16}N_4O_3$		3
	Trimeth-276b	276.1222		$C_{13}H_{16}N_4O_3$		3
	Trimeth-294a	294.1328	5.0	$C_{13}H_{18}N_4O_4$		3
	Trimeth-294b	294.1328		$C_{13}H_{18}N_4O_4$		3

Metronidazole	Metronid-185a	185.0437	2.3	$C_6H_7N_3O_4$		3
Metronidazole	Metronid-185b	185.0437	2.3	$C_6H_7N_3O_4$		3
	Metronid-185c	185.0437		$C_6H_7N_3O_4$		3
	Metronid-185d	185.0437		$C_6H_7N_3O_4$		3

Cefadroxil	Cefa-217	216.0529	2.0	$C_{11}H_8N_2O_3$	-	4
	Cefa-233	232.0250	2.0	-	-	5
Sulfamethoxazole	Sulfa-340	339.0883	6.7	$C_{14}H_{17}N_3O_5S$	-	4
	Sulfa-342	341.1253	5.3	-	-	5

The application of both suspect and non-target screening for TRI resulted to the tentative identification of 15 TPs. According to the results, two were the main degradation pathways for this compound; the one begins with hydroxylation and takes place during both phyto- and photodegradation, while the other begins with demethylation and occurs only in absence of *L. minor* (Figure 3.4.3). It is worth mentioning that 3 peaks were detected (3 isomers) for the suspect TP Trimeth-306 for both phyto- and photodegradation, with the difference that in phytodegradation there is a clear preference for a specific hydroxylation position, while in photodegradation, this is not the case (Figure S3.4.2). This shows that this specific isomer is part of a *L. minor's* metabolic pathway (biological specificity), which is not valid for abiotic processes such as photodegradation.

Through suspect and non-target screening of METRO samples, four candidate TPs were tentatively identified (Figure 3.4.4). Two degradation pathways of METRO were proposed, which involve oxidation and hydroxylation reactions (Figure 3.4.4).

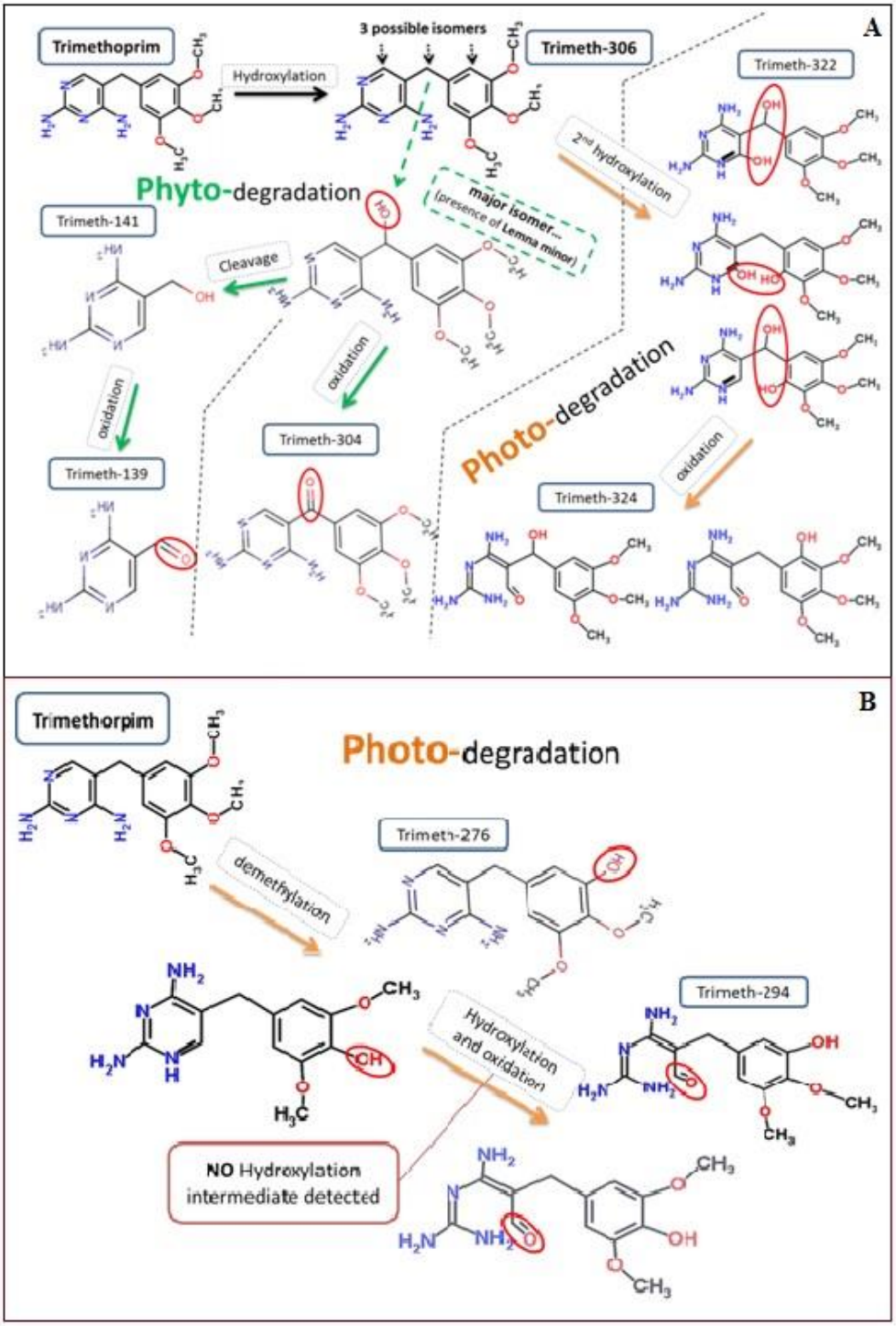


Figure 3.4.3 Proposed degradation pathways for trimethoprim (TRI) in the presence (a) and absence of *L. minor* (b).

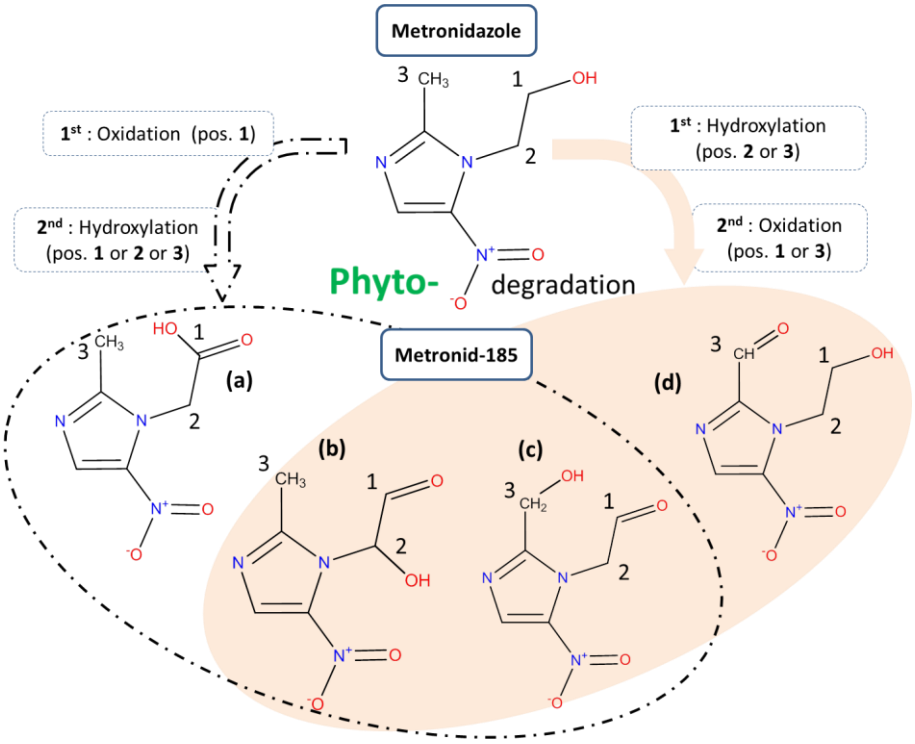


Figure 3.4.4 Degradation pathways of metronidazole (METRO). The one pathway (left) starts with an oxidation reaction, followed by a hydroxylation in 3 candidate positions. The other pathway (right) consists of a hydroxylation followed by an oxidation.

Regarding the toxicity of parent antimicrobials and their TPs, LC50 values of TRI and METRO as well as their tentative identified TPs were estimated with in-house QSAR models, built and validated with novel chemometrics (Table S3.4.5). The majority of TPs shown comparable LC50 values to their parent compounds. It is worth noting that in some cases toxicity of TPs was significantly higher (e.g. Trimeth-141, Trimeth-139). Nevertheless, since quantitative data concerning TPs are not available, assessment of their ecotoxicological risk is not possible.

Two TPs were also detected and an unequivocal molecular formula for one of them was proposed, for both SMX and CFD. The TPs of SMX were sulfameth-340, with a proposed formula C14H17N3O5S, and sulfameth-342, while the TPs of CFD were cefa-217, with a proposed formula C11H8N2O3, and cefa-233. Since TPs of CFD were coeluting, their elution profiles, MS and MS/MS spectra were checked in order

to investigate whether cefa-217 was in-source fragment of cefa-233. We concluded that they were two different chromatographic peaks.

3.4.3.4 Removal of target antimicrobials in continuous – flow experiments

To investigate the potential of continuous-flow *L. minor* system to remove antimicrobials, a lab-scale system consisting of three mini ponds in series was used and METRO and TRI were used as models. Aerobic conditions existed in all ponds, while pH values presented a slight increase between Pond 1 and Pond 3 (Table S3.4.6) due to plants' photosynthetic activity (Ran et al., 2004; Priya et al., 2012). The performance of the duckweed system was satisfactory, achieving the limits for COD, TN and TP set by the European Directive for wastewater discharge to the aquatic environment (EC, 1991). The average TP and NH_4-N removal was equal to 81 ± 2% and 96 ± 1%, respectively (Table S3.4.7), while the removal of NO_3-N was lower (28 ± 2%), indicating the preference of duckweeds to remove nitrogen in the form of ammonia (Ran et al., 2004; Iatrou el al., 2015). The growth of *L. minor* was not affected by the addition of micropollutants and weekly harvesting of the biomass was conducted to maintain a density of 600 g fresh weight per m^2.

Regarding micropollutants, the existence of different ponds in series resulted to a gradual decrease of their concentrations (Table S3.4.8). As a result, a total removal of 71 ± 11% and 61 ± 8% was observed at the outlet of the system for METRO and TRI, respectively (Figure 3.4.5). This is the first study reporting antimicrobials removal in duckweed continuous-flow wastewater treatment systems. The achieved removal is comparable with those observed in other constructed wetland systems. Specifically, the removal of TRI was studied in constructed wetlands planted with *Typha angustifolia* and *Phragmites australis* and ranged between 65 and 96% (Hijosa-Valsero et al., 2011). In another study, TRI was removed by 35 to 97% in vertical subsurface-flow, surface-flow and horizontal subsurface-flow systems planted with *Thalia dealbata* and *Arundo donax* (Dan et al., 2013). Finally, Li et al. (2014) reported that METRO was fully eliminated in a horizontal subsurface flow bed planted with *Phragmites australis*.

To estimate the contribution of different mechanisms on the removal of target antimicrobials in continuous-flow system, batch kinetics constants were used for

hydrolysis, photodegradation, sorption and plant uptake (Table 3.4.2) and Equations 3.4.6 and 3.4.7 were applied. As the continuous-flow system operated on non-sterilized conditions treating real secondary treated wastewater, it is possible that a part of target compounds could also be removed via biodegradation by bacteria found in wastewater or/and by the biofilm developed on the surface of the plant roots. To quantify the mechanism of biodegradation, a literature review was conducted for the target compounds and the biodegradation constants recently calculated in an aerobic stabilization pond (0.06 d^{-1} for METRO; 0.0092 d^{-1} for TRI) were used (Møller et al., 2016).

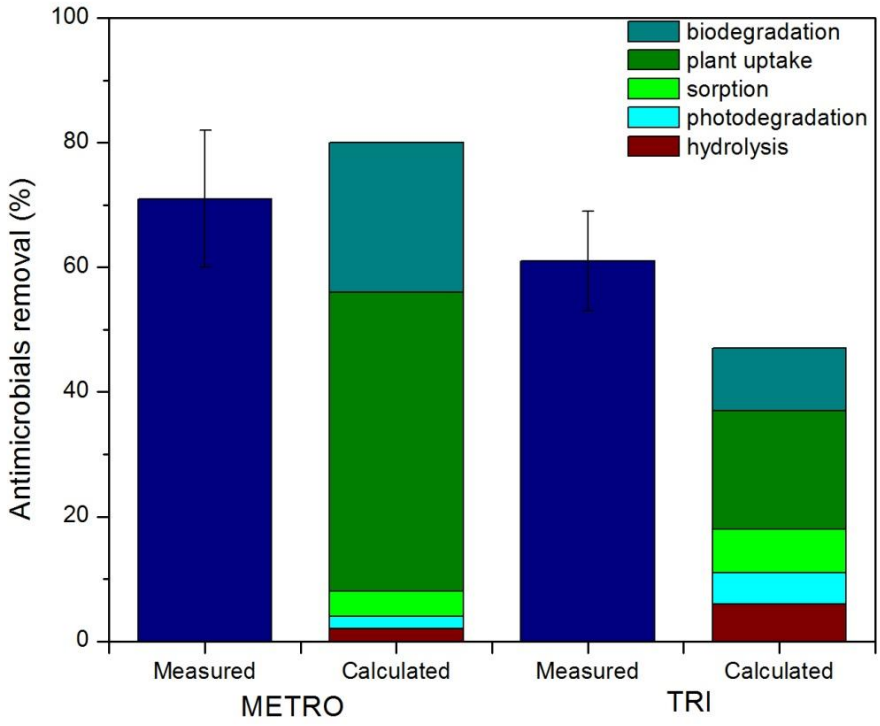


Figure 3.4.5 Measured and predicted removal of metronidazole (METRO) and trimephoprim (TRI) in *L. minor* continuous-flow system. The contribution of different mechanisms on their removal is also shown (for predicted removal, the removal due to hydrolysis, photodegradation, sorption to biomass, plant uptake and biodegradation were calculated).

The applied model described quite sufficiently the removal of studied micropollutants in continuous-flow system, resulting to total removal of 80% for METRO and 47% for TRI (Figure 3.4.5). Concerning the contribution of different mechanisms to the target compounds removal, it seems that plant uptake and biodegradation were the major mechanisms governing METRO removal, being responsible for 48% and 20% of the total removal respectively, while the effect of the other mechanisms was of minor importance. Regarding TRI, the contribution of all mechanisms was much more balanced, while the most important mechanism was plant uptake being responsible for 19% of the observed total removal.

4 Conclusions and future research

4.1 Conclusions

The collection of data for the consumption of antimicrobials in Greece showed that the higher sales of antimicrobials were observed for amoxicillin, clarithromycin, cefuroxime axetil, ciprofloxacin and cefaclor. The highest PECs for raw wastewater were estimated for amoxicillin (27 μg L^{-1}) and clarithromycin (8.1 μg L^{-1}), while for treated wastewater for cefuroxime axetil (6.6 μg L^{-1}) and clarithromycin (4.5 μg L^{-1}). Appication of Risk Quotient (RQ) methodology showed that between the studied aquatic organisms (algae, daphnids, fish), a significant ecotoxicological threat due to the presence of antimicrobials was estimated for algae. RQ values higher than 100 were calculated for amoxicillin, clarithromycin and ciprofloxacin in raw and treated wastewater (acute toxicity data) and amoxicillin, clarithromycin (chronic toxicity data). The results of this study revealed that the release of human antimicrobials to the aquatic environment through treated wastewater disposal may potentially be a significant environmental threat, especially for rivers with low to moderate dilution.

The batch experiments that studied the growth of *Lemna minor* in human urine (HU) and wastewater showed that the cultivation of *L.minor*in diluted HU or treated wastewater is possible achieving significant removal of major pollutants, efficient elimination of SMX and production of biomass with high starch for possible use as biofuel. For both test media, removal of urea, COD, TP, TN, NH_4^+-N, SMX and CIP exceeded 84%, 83%, 94%, 50%, 58%, 82% and 88%, respectively. The major mechanisms governing SMX and CIP removal were plant uptake and photodegradation, respectively. The higher starch content (47.1%) was achieved when biomass was cultivated in HU for 7 d and then transferred to tap water for 21 d.

The experiments that investigated the biomass production in wastewater treatment systems planted with *L.minor*, *L.gibba* or combination of the two duckweeds showed that the highest biomass production was achieved in ponds planted with *L. minor* and *L. gibba* (System 3). The addition of 30 mg L^{-1} NH_4-N resulted to significant increase of protein content in all experimental systems, while the highest protein contentand the highest protein productivity were observed in System 3 and they were equal to

44.4% and 8.1 g m^{-2} d^{-1}, respectively. The transfer of duckweeds in water containing no nutrients for a period of 21 days increased their starch content. Percentages ranging between 43.9% (*L. minor*) to 46.1% (*L. minor + L. gibba*) were observed; whereas starch productivity reached 8.1 g m^{-2} d^{-1} at System 3. The application of this system as a polishing stage in municipal wastewater treatment seems to combine low operating costs, sufficient conventional pollutants' removal and production of biomass that can be used as feedstock or for bioethanol production.

Finally, the toxicity experiments with CFD, METRO, TMP and SMX showed that no effect was noticed on duckweed specific growth rates for concentrations of CFD and METRO up to 10000 μg L^{-1} and for concentrations of TMP and SMX up to 2000 μg L^{-1}. In batch experiments, the presence of active *L. minor* decreased significantly the half-life values of tested antimicrobials, ranging from 2.5 ± 0.1d (CFD) to 20 ± 0.8d (TRI). The application of both suspect and non-target screening for TRI resulted to the tentative identification of 15 transformation products (TPs). Two were the main degradation pathways for this compound; the one begins with hydroxylation and takes place during both phyto- and photodegradation, while the other begins with demethylation and occurs only in absence of *Lemna minor*.Additonally, four candidate TPs were tentatively identified for METRO while two degradation pathways were proposed, which involve oxidation and hydroxylation reactions.The continuous-flow experiments showed that the performance of the duckweed system was satisfactory, achieving the limits for COD, TN and TP set by the European Directive for wastewater discharge to the aquatic environment. Regarding micropollutants, the existence of different ponds in series resulted to a gradual decrease of their concentrations and a total removal of 71 ± 11% and 61 ± 8% for METRO and TRI, respectively. The plant uptake was the major mechanism governing target compounds' removal.

4.2 Future Research

According to the results of the current PhD Dissertation, the following points are suggested for future research.

As RQ values seem to be mainly affected by available PNEC data, future efforts should be focused on estimating acute and chronic toxicity of antimicrobials to different species of aquatic organisms. The experimental estimation of acute toxicity of antimicrobials in mixture is also a crucial step for understanding their mode of action on aquatic organisms.

As urine consists an inexpensive source of nutrients, the future installation of source-separating toilets constitutes a possible solution for their efficient recovery. Further research and optimization of the process are required for the large-scale production of duckweed biomass from human urine. A thoroughly characterization of biomass is also needed to assure safety of the products due to the presence of pathogens and different groups of micropollutants.

The results of batch and continuous - flow experiments showed that *Lemna minor* bioreactors could be used in the future for the simultaneous removal of major pollutants and pharmaceuticals from wastewater, achieving in parallel important production of biomass with high protein or starch content. Further research is needed on the role of plant uptake on micropollutants' removal as well as on the transformation products formed due to different involved biotic and abiotic processes. More information is also needed for the characterisitcs of the produced biomass in order to find the optimal options for its valorization. Finally, studies for the removal efficiency of antimicrobial resistance genes (ARGs) in *Lemna minor* systems are also needed.

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6 Supplementary and Materials

6.1 Supplementary material for section 3.1

6.1.1 Tables

Table S3.1.1 Excretion rates (ER) ofantimicrobials in urine and removal efficiencies (R) during conventional biological wastewater treatment.

Antimicrobials	Excretion Rate, ER (%)	Removal Efficiency, R (%)
Amoxicillin	49.5[1]	89.0[23]
Clarithromycin	35.0[2]	45.0[24]
Ciprofloxacin	53.1[3]	66.0[25]
Cefaclor	72.5[4]	93.8[23]
Cefprozil	65.0[5]	22.0[26]
Azithromycin	6.0[6]	49.0[24]
Metronidazole	50.0[7]	39.0[27]
Norfloxacin	30.0[8]	57.0[28]
Sulfamethoxazole	9.5[9]	74.0[25]
Erythromycin	4.0[10]	49.0[24]
Netilmicin	80.0[11]	22.0[26]
Loracarbef	92.0[12]	22.0[26]
Floxacin	84.3[13]	40.0[25]
Ceftriaxone	66.0[14]	51.0[28]
Cefadroxil	88.0[15]	22.0[26]
Meropenem	66.5[16]	75.1[26]
Cefuroxime Axetil	60.0[17]	8.8[26]
Clindamycin	25.5[18]	27.0[28]
Doxycycline	70.0[19]	61.0[23]
Cefuroxime	60.0[17]	22.0[26]
Levofloxacin	38.4[20]	42.0[29]

Amikacin	25.0[21]	92.1[26]
Moxifloxacin	20.0[22]	0[26]
Trimethoprim	60.0[9]	30.0[28]

Table S3.1.2 Physicochemical properties of target antimicrobials.

Antibiotics	**LogK$_{ow}$ (25 ºC)**	**Water Solubility (mg L^{-1}, 25 ºC)**	**Melting Point (25 ºC)**
Amoxicillin	0.87[1]	4000[1]	330[4]
Azithromycin	4.02[1]	0.062[3]	110[1]
Cefaclor	0.35[2]	10000[1]	330[4]
Cefadroxil	0[2]	1100[3]	330[4]
Cefprozil	0.69[2]	3500[3]	340[4]
Ceftriaxone	-1.9[2]	790[3]	350[4]
Ciprofloxacin	0.28[1]	1.1[1]	320[4]
Clarithromycin	3.16[1]	0.34[3]	220[1]
Erythromycin	3.06[1]	0.52[3]	190[1]
Loracarbef	0.4[2]	2800[3]	320[4]
Meropenem	-1.2[2]	2200[3]	330[4]
Metronidazole	0[1]	9500[1]	160[1]
Netilmicin	-2.4[2]	10000[3]	270[4]
Norfloxacin	-1[1]	180000[3]	230[1]
Ofloxacin	-0.3[1]	28000[3]	250[1]
Sulfamethoxazole	0.89[1]	610[1]	170[1]
Trimethoprim	0.91[1]	400[1]	201[1]
Cefuroxime axetil	0.89[1]	106.6[3]	291.35[1]
Cefuroxime	-0.16[1]	144.8[3]	289.4[4]
Doxycycline	-0.02[1]	312.9[1]	331.01[4]
Clindamycin	2.16[1]	30.61[3]	255.26[4]

Levofloxacin	-0.3[1]	28000[3]	250[1]
Amikacin	-8.78[2]	1.85E+05[1]	204[1]
Moxifloxacin	0.95[2]	7450[3]	325[4]
[1] Exper. database match from EPI, [2]KOWWIN v1.67 estimate, [3]WSKOW v1.41 estimate, [4]MPBPVP v1.43 estimate			

Table S3.1.3 Acute toxicity data for the target compounds and different aquatic organisms.

Antibiotics	**Aquatic Organisms**	**Exposure Time**	**EC_{50}/LC_{50} (mgL^{-1})**
Amoxicillin	Fish	96 h	2544.969[1]
	Daphnia	48 h	1281.025[1]
	Algae	96 h	0.00222[2]
Clarithromycin	Fish	48 h	12.21[3]
	Daphnia	24 h	25.72[3]
	Algae	96 h	0.012[4]
Azithromycin	Fish	96 h	11.268[1]
	Daphnia	48 h	120[5]
	Algae	96 h	0.019[4]
Ciprofloxacin	Fish	96 h	7285.346[1]
	Daphnia	48 h	3414.681[1]
	Algae	96 h	0.005[6]
Metronidazole	Fish	96 h	6751.782[1]
	Daphnia	48 h	3051.886[1]
	Algae	72 h	38.8[7]
Norfloxacin	Fish	96 h	90144.031[1]
	Daphnia	48 h	36063.004[1]
	Algae	N.A.	10.4[8]

Sulfamethoxazole	Fish	96 h	562.5[9]
	Daphnia	7 days	0.21[3]
	Algae	96 h	0.03[10]
Loracarbef	Fish	96 h	5969.644[1]
	Daphnia	48 h	963[5]
	Algae	96 h	638.169[1]
Erythromycin	Fish	48 h	0.94[3]
	Daphnia	24 h	22.45[3]
	Algae	72 h	0.02[3]
Ofloxacin	Fish	48 h	0.53[3]
	Daphnia	72 h	1.44[3]
	Algae	96 h	0.016[11]
Cefaclor	Fish	96 h	7055.903[1]
	Daphnia	48 h	3335.243[1]
	Algae	96 h	730.63[1]
Cefprozil	Fish	96 h	3851.68[1]
	Daphnia	48 h	1897.034[1]
	Algae	96 h	477.786[1]
Netilmicin	Fish	96 h	2.35E+06[1]
	Daphnia	48 h	7.88E+05[1]
	Algae	96 h	53639.285[1]
Cefadroxil	Fish	96 h	16112.755[1]
	Daphnia	48 h	7230.523[1]
	Algae	96 h	1327.742[1]
Ceftriaxone	Fish	96 h	1.02E+06[1]
	Daphnia	48 h	3.62E+05[1]
	Algae	96 h	30360.141[1]
Meropenem	Fish	96 h	1.66E+05[1]
	Daphnia	48 h	6.47E+03[1]
	Algae	96 h	7355.32[1]
Trimethoprim	Fish	96 h	1870.43[1]
	Daphnia	48 h	92[10]

	Algae	96 h	80.39696[8]
Cefuroxime axetil	Fish	96 h	3419.477[1]
	Daphnia	48 h	1725.38[1]
	Algae	96 h	471.718[1]
Clindamycin	Fish	96 h	239.709[1]
	Daphnia	48 h	141.021[1]
	Algae	96 h	64.923[1]
Doxycycline	Fish	96 h	241000[1]
	Daphnia	48 h	92495.672[1]
	Algae	96 h	10026.92[1]
Cefuroxime	Fish	96 h	21991.492[1]
	Daphnia	48 h	9773.602[1]
	Algae	96 h	1736.771[1]
Levofloxacin	Fish	96 h	20236.246[1]
	Daphnia	48 h	8950.357[1]
	Algae	5 days	0.0079[12]
Amikacin	Fish	96 h	5.98E+11[1]
	Daphnia	48 h	93700000000[1]
	Algae	96 h	484000000[1]
Moxifloxacin	Fish	96 h	2383.55[1]
	Daphnia	48 h	1211.71[1]
	Algae	96 h	339.803[1]

Table S3.1.4 Chronic toxicity data for the target compounds and different aquatic organisms.

Antibiotics	**Aquatic Organisms**	**Exposure Time**	**NOEC/LOEC (mgL^{-1})**
Amoxicillin	Fish	7 days	0.1[1]
	Daphnia	-	-
	Algae	96 h	0.00078[2]

Ciprofloxacin	Fish	96 h	100[3]
	Daphnia	24 h	60[3]
	Algae	-	-
Metronidazole	Fish	14 days	10[4]
	Daphnia	21 days	250[5]
	Algae	-	-
Loracarbef	Fish	-	-
	Daphnia	-	-
	Algae	24 h	13[6]
Clarithromycin	Fish	-	-
	Daphnia	-	-
	Algae	96 h	0.0052[7]
Norfloxacin	Fish	-	-
	Daphnia	-	-
	Algae	-	4.02[8]
Ofloxacin	Fish	48 h	12.5[9]
	Daphnia	-	-
	Algae	96 h	0.005[10]
Trimethoprim	Fish	72 h	100[3]
	Daphnia	21 days	6[11]
	Algae	96 h	25.5[8]

-No experimental data is available

Table S3.1.5 Average dilution factors (D) for Sewage Treatment Plants (STPs) discharging treated wastewater to Greek rivers.

Sewage Treatment Plant	River	Dilution Factor (D)
Ptolemaida	Soulou	3
Katerini	Aisonas	11
Trikala	Lithaios	11

Leivadia	Erkynas	14
Florina	Sakoulevas	15
Komotini	Vozvozis	16
Sparti	Evrotas	18
Drama	Aggitis	22
Ioannina	Kalamas	49
Karditsa	Peneios	101
Karpenisi	Karpenisiotis	133
Larisa	Peneios	142
Giannitsa	Loudias	230
Kalampaka	Pineios	273
Serres	Strymonas	286
Pyrgos	Alfeios	318
Veroia	Aliakmonas	608
Tyrnavos	Titarisios	750
Kilkis	Gallikos	790
Agrinio	Aheloos	824
Arta	Arahthos	873
Kastoria	Aliakmonas	913
Krestena	Alfeios	1910
Orestiada	Evros	2388

Table S3.1.6 Average dilution factors (D) for Sewage Treatment Plants (STPs) discharging treated wastewater to Greek rivers.

Sewage Treatment Plant	River	Dilution Factor (D)
Ptolemaida	Soulou	3
Katerini	Aisonas	11
Trikala	Lithaios	11
Leivadia	Erkynas	14
Florina	Sakoulevas	15

Komotini	Vozvozis	16
Sparti	Evrotas	18
Drama	Aggitis	22
Ioannina	Kalamas	49
Karditsa	Peneios	101
Karpenisi	Karpenisiotis	133
Larisa	Peneios	142
Giannitsa	Loudias	230
Kalampaka	Pineios	273
Serres	Strymonas	286
Pyrgos	Alfeios	318
Veroia	Aliakmonas	608
Tyrnavos	Titarisios	750
Kilkis	Gallikos	790
Agrinio	Aheloos	824
Arta	Arahthos	873
Kastoria	Aliakmonas	913
Krestena	Alfeios	1910
Orestiada	Evros	2388

6.1.2 Figures

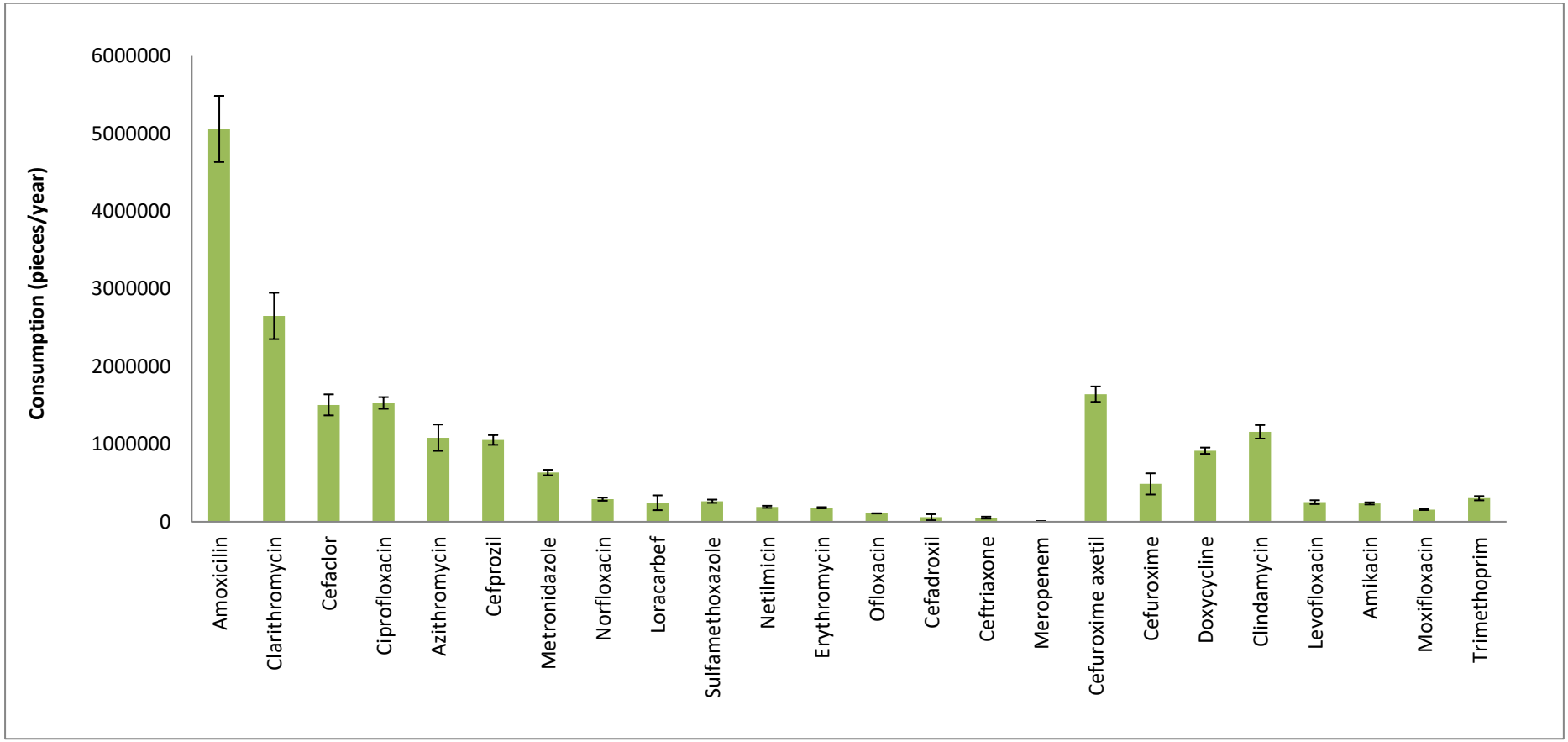

Figure S3.1.1 Average annual consumption of antimicrobials (as pieces per year) in Greece for years 2008-2010

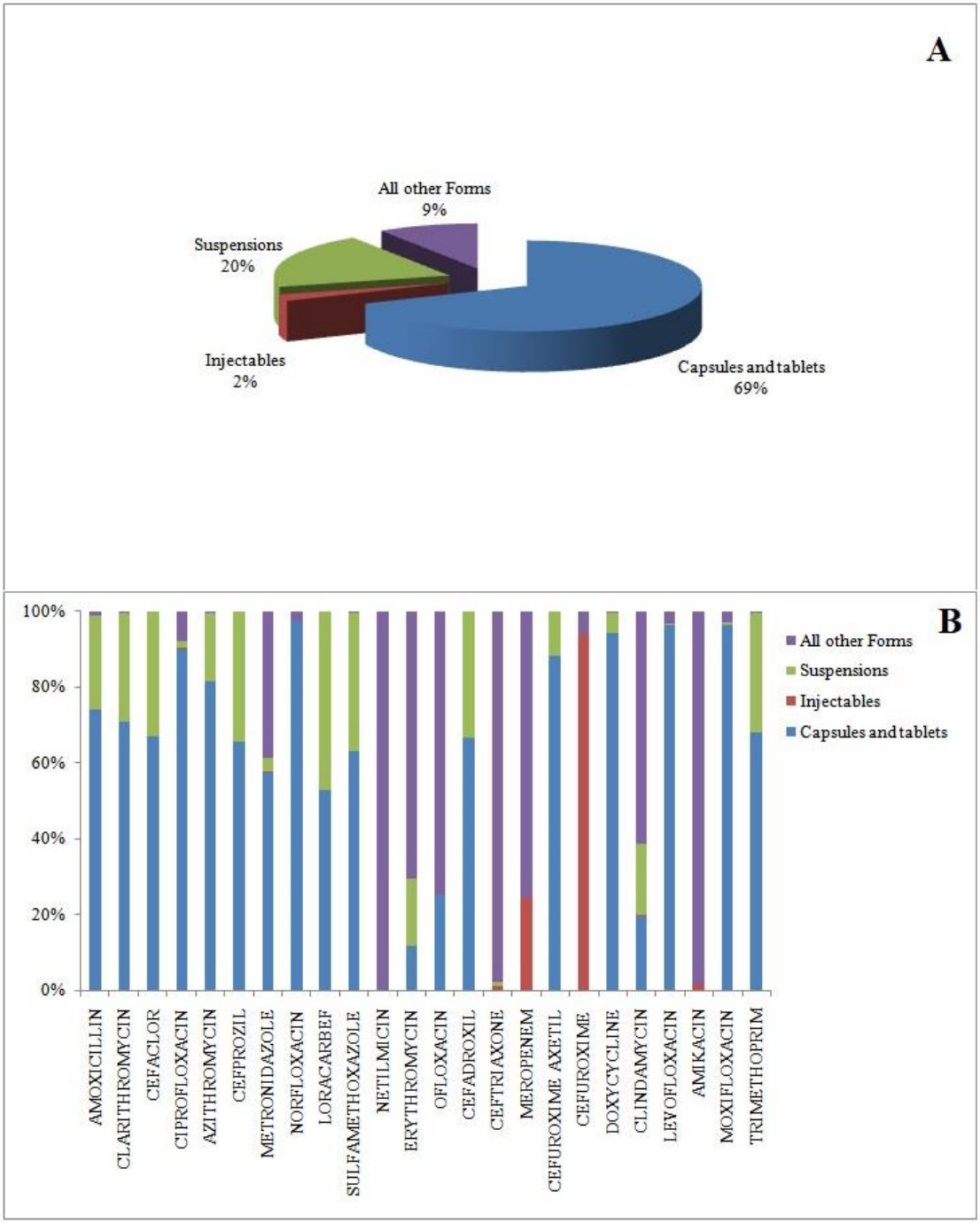

Figure S3.1.2 Sales per form of medication for (a) the 24 studied antimicrobials and (b) each antimicrobial separately.

6.1.3 References

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[8] Eguchi K., Nagase H., Ozawa M.,.Endoh Y. S, Goto K., Hirata K., Miyamoto K. and Yoshimura H. (2004) Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57:1733-1738

[9] Kim S. and Aga D.S. (2007) Potential Ecological and Human Health Impacts of Antibiotics and Antibiotic-Resistant Bacteria from Wastewater Treatment Plants. Journal of Toxicology and Environmental Health, Part B 10:559–573

[10] Park S. and Choi K. (2008) Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology 17:526-538

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12 Robinson AA, Belden JB, Lydy MJ (2005) Toxicity of flouroquinolone antibiotics to aquatic organisms. Environmental Toxicology and Chemistry 24:423–430

Table S3.1.4:

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4 Khalil W. K. B., Mahmoud M. A., Zahran M. M. and Mahrous K.F. (2007) A sub-acute study of metronidazole toxicity assessed in Egyptian Tilapia zillii. Journal of applied toxicology 27:380-390

5 Wollenberger L., Halling-Sorensen B. and Kusk K. O. (2000) Acute and chronic toxicity of veterinary antibiotics to Daphnia magna, Chemosphere 40:723-730

6 Crane Mark, Watts Chris, Boucard Tatiana (2006) Chronic aquatic environmental risks from exposure to human pharmaceuticals. Science of the Total Environment 367:23-41

7 Harada A., Komori K., Nakada N., Kitamura K. and Suzuki Y. (2008) Biological effects of PPCPs on aquatic lives and evaluation of river waters. Water Science & Technology 58:1541-1546

8 Eguchi K., Nagase H., Ozawa M., Endoh Y. S., Goto K., Hirata K., Miyamoto K. and Yoshimura H. (2004) Evaluation of antimicrobial agents for veterinary use in the ecotoxicity test using microalgae. Chemosphere 57:1733-1738

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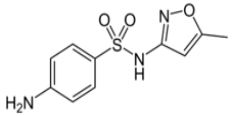
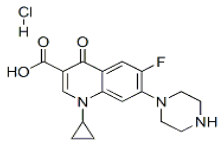
[11] Park S. and Choi K. (2008) Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology 17:526-538

6.2 Supplementary material for section 3.2

6.2.1 Tables

Table S3.2.1 Chemical structures and physicochemical properties of studied antimicrobials.

Antimicrobial	Category	Formula	Structure	MW[a]	S_w[b]	$LogK_{ow}$[c]
Sulfamethoxazole	sulfonamides	$C_{10}H_{11}N_3O_3S$		253.3	3942	0.89
Ciprofloxacin	quinolones	$C_{17}H_{19}ClFN_3O_3$		367.8	30000	0.28

[a]MW: molecular weight; [b]S_w: solubility at 25°C (mg L^{-1}); [c]$LogK_{ow}$: octanol-water partition coefficient

Structure and physicochemical data source: ChemIDplus Advanced (http://chem.sis.nlm.nih.gov/chemidplus/), ChemSpider(http://www.chemspider.com/), DrugBank (http://www.drugbank.ca/drugs).

Table S3.2.2 Composition of the Swedish standard (SIS) sterile growth medium.

Substance	Concentration in medium solution (g L^{-1}) *
$NaNO_3$	85
KH_2PO_4	13.4
$MgSO_4 \cdot 7H_2O$	75
$CaCl_2 \cdot 2H_2O$	36
$NaCO_3$	20
H_3BO_3	1
$MnCl_2 \cdot 4H_2O$	0.2
$NaMoO_4 \cdot 2H_2O$	0.01
$ZnSO_4 \cdot 7H_2O$	0.05
$CuSO_4 \cdot 5H_2O$	0.005
$Co(NO_3)_2 \cdot 6H_2O$	0.01
$FeCl_3 \cdot 6H_2O$	0.84
Na_2 – EDTA $2H_2O$	1.4

*(OECD, 2006)

Table S3.2.3 Composition of the synthetic urine (SU).

Substance	Concentration (g L^{-1})*
Urea	10.72
NaCl	4.83
K_2HPO_4	4.12
Na_2SO_4	2.37
Creatine	1
$MgCl_2 \cdot 6H_2O$	0.85
Sodium citrate	0.65
$CaCl_2$	0.38
KCl	0.29

*(Feng and Wu, 2006; Chang et al., 2013)

Table S3.2.4 Concentrations (mean ± sd) of the major pollutants in experiments with duckweed *Lemna minor* cultivating in human urine (stored for 1 d; dilution factor: 1:200) and secondary treated wastewater (duration of the experiment: 14 d; initial mass of duckweed: 1.5 g; temperature: 24˚C; pH: 7)

	Human Urine (HU)[1]				**Human Urine (HU) & harvesting[2]**				**Secondary treated wastewater (WW)[3]**			
Day	**0**	**5**	**10**	**14**	**0**	**5**	**10**	**14**	**0**	**5**	**10**	**14**
Urea, mg L^{-1}	41.4 ± 0.5	26.8 ± 0.2	8.7 ± 0.2	6.5 ± 0.0	41.4 ± 0.5	23.2 ± 0.0	6.7 ± 0	4.2 ± 0.1	4.4 ± 0.1	2.4 ± 0.1	1.1 ± 0.1	0.1 ± 0.1
TP, mg L^{-1}	5.2 ± 0.1	3.1 ± 0.1	-	0.3 ± 0	5.2 ± 0.1	3.0 ± 1.0	-	0.3 ± 0.0	4.8 ± 0.2	2.0 ± 0.1	-	0.2 ± 0.0
TN, mg L^{-1}	34.0 ± 1.0	-	-	17.0 ± 0.6	34.0 ± 1.0	-	-	16.7 ± 0.6	43.7 ± 0.6	-	-	13.3 ± 0.6
COD, mg L^{-1}	52.3 ± 2.5	36.7 ± 1.5	23.0 ± 1.0	2.1 ± 0.3	52.3 ± 2.5	36.7 ± 1.5	23.0 ± 1	2.1 ± 0.3	45.3 ± 0.6	33.3 ± 1.5	17 ± 1.0	7.6 ± 0.4
NH_4^+-N, mg L^{-1}	2.4± 0.1	6.5 ± 0.1	3.5 ± 0.1	1.1 ± 0.0	2.4± 0.1	6.2 ± 0.0	2.9 ±0.1	1.0 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0	0

[1]HU: human urine; [2]HU& harvesting: human urine with harvested biomass at Days 5 and 10; [3]WW: secondary treated wastewater

6.2.2 Figures

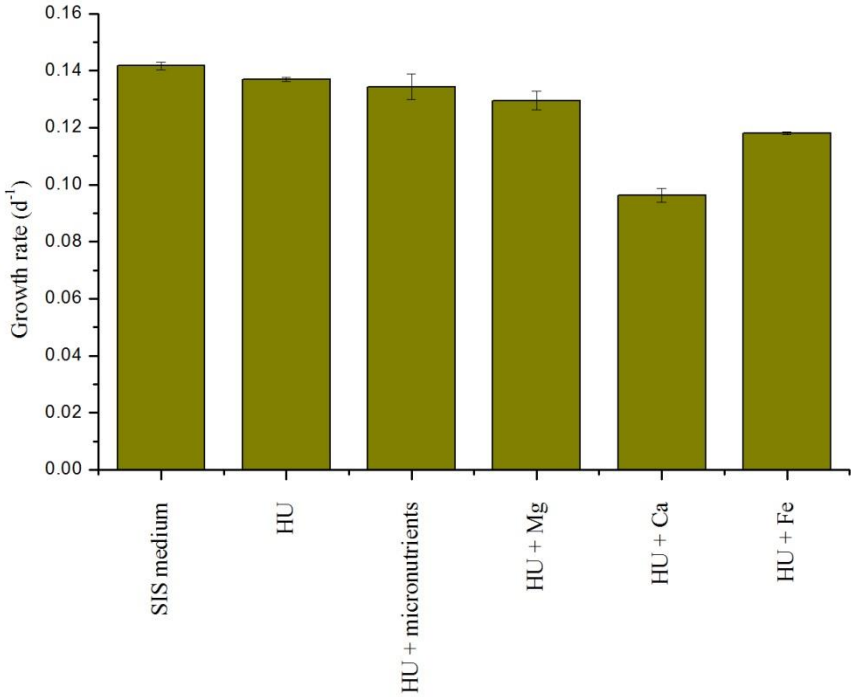
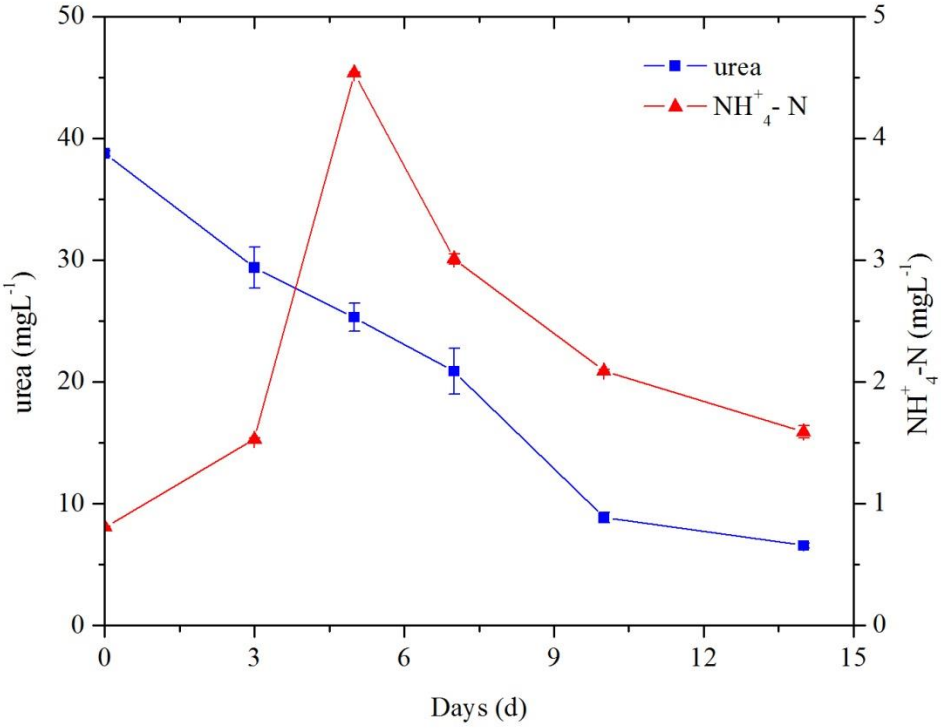


Figure S3.2.1 Growth rates, μ (d^{-1}) for experiments with addition of macro/microelements (SIS medium: control medium; HU: human urine; HU + micronutrients: HU and mixture of: B, Mn, Mo, Zn, Cu, Co; HU + Mg: HU and Mg; HU + Ca: HU and Ca; HU + Fe: Human urine and Fe). (HU stored for 1 d and dilution factor: 1:200; duration of the experiment: 10 d; initial mass of duckweed: 0.5 g; temperature: 24˚C; pH: 7; growth rates were calculated using Equation 3.2.1).



FigureS3.2.2 Concentration changes of NH_4^+-N and urea in human urine (HU) diluted by a factor of 1:200 (duration of the experiment: 14 d; initial mass of duckweed: 1.5 g; temperature: 24˚C; pH: 7)

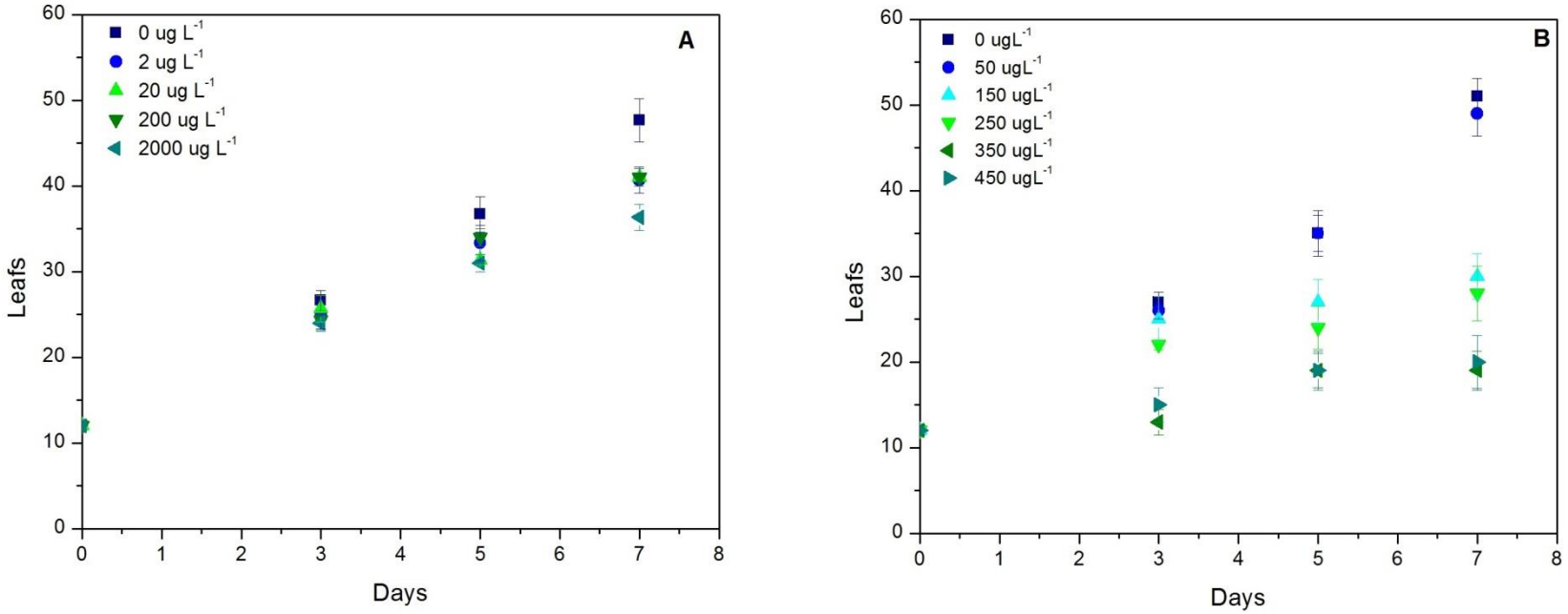


Figure S3.2.3 Toxicity range tests for sulfamethoxazole, SMX (A) and ciprofloxacin, CIP (B)

6.3 Supplementary material for section 3.3

6.3.1 Tables

Table S3.3.1 Characteristics of the duckweeds used in the current study, *L.minor* and *L.gibba.*

Kingdom	Plantae	
Subkingdom	Tracheobionta (vascular plants)	
Superdivision	Spermatophyta (seed plants)	
Division	Magnoliophyta (flowering plants)	
Class	Liliopsida (monocotyledons)	
Subclass	Arecidae	
Order	Arales	
Family	Lemnaceae (duckweed family)	
Sub-family	Lemnoideae	
Genus	*Lemna L.* (duckweed)	
Species	*L.minor*	*L.gibba*
Common names	Lesser duckweed	Swollen duckweed

Table S3.3.2 Composition of Swedish standard (SIS) sterile growth medium.

The same Table as in Section 6.2 (Table S3.2.2).

Table S3.3.3 Experimental conditions during Phases A and B in different Systems.

System	Initial biomass (g)	Tank volume (L)	Media	Hydraulic Retention Time, HRT (days)	Duration of Phase A [5] (days)	Duration of Phase B [6] (days)
1	13[1]	5	Wastewater [4]	4	16	16
2	13[2]	5	Wastewater [4]	4	16	16
3	13[3]	5	Wastewater [4]	4	16	16

[1]*Lemnaminor*, [2]*Lemnagibba*, [3] Combination *L.minor*and*L.gibba* (6.5g + 6.5g), [4] Secondary treated wastewater,[5] No addition of NH_4-N, [F6]Addition 30 mg/LNH_4-N

Table S3.3.4 Experimental conditions during Phase C in different Systems.

System	Initial biomass (g)	Volume (ml)	Media	Duration (days)	Light
1	2^1	100	Tap water	21	Yes
2	2^2	100	Tap water	21	Yes
3	2^3	100	Tap water	21	Yes

At the end of Phase B (Day 32), biomass was transferred from each experimental setup:

[1]*L. minor*, [2]*L. gibba*, [3]Combination *L.minor*and*L.gibba*

6.4 Supplementary material for section 3.4

6.4.1 Analytical method used for transformation products identification

For the chromatographic separation, a Thermo Dionex Acclaim RSLC C18 column (2.2 μm, 120 Å, 2.1×100 mm), thermostated at 30 ^{o}C, was used. The chromatographic run lasted 15.5 min with 5 min of re-equilibration of the column to the initial conditions of the mobile phase before the next injection. The mobile phases were H_2O:MeOH (90:10) (solvent A) and MeOH (solvent B) both modified with 0.01% formic acid and 5 mM ammonium formate. The gradient elution program started with 1% B with a flow rate of 0.2 mL min^{-1} for 1 min and it increased to 39% in 2 min (flow rate 0.2 mL min^{-1}), and then to 99.9% (flow rate 0.4 mL min^{-1}) in the following 11 min. Then it was kept constant for 2 min (flow rate 0.48 mL min^{-1}) and then initial conditions were restored within 0.1 min and the flow rate decreased to 0.2 mL min^{-1}. The injection volume was set up to 5 μL.

The QTOF spectrometer was equipped with electrospray ionization interface (ESI) operating in positive ionization mode, with the following operation parameters: capillary voltage, 2500 V; end plate offset, 500 V; nebulizer pressure, 2 bar (N2); drying gas, 8 L min−1 (N2); and drying temperature, 200 °C. Data acquisition was performed through broad-band collision induced dissociation (bb-CID), switching between 4eV (low collision energy, LE) and 25eV (high collision energy, HE) in the collision cell Q2, providing both MS and MS/MS spectra simultaneously, within a mass-to-charge (m/z) range of 50–1000 for each sample, at 2 Hz spectra rate. The QTOF was daily external calibrated with a sodium formate solution mixture, consisted of 10 mM sodium formate in a mixture of H2O/isopropanol (1:1). At the beginning of each chromatographic run an internal calibration was performed using a calibrant injection, at a segment of 0.1-0.25 min.

For the detection and identification of tentative TPs, both suspect and non-target screening workflows were applied. Regarding suspect screening, samples were screened by extracting the exact masses of the potential TPs, according to a suspect database established for each compound. To accomplish that, in-silico metabolite/transformation/degradation prediction tools, such as the online pathway prediction system hosted by EAWAG institute (EAWAG-PPS) and MetabolitePredict software (BrukerDaltonik, Bremen, Germany), were applied. EAWAG-PPS was

performed to predict two generations of TPs for each compound. MetabolitePredict was used to predict possible metabolites according to phase I, II and CYP450 metabolism rules, thereby including rules such as hydroxylation (missing from the EAWAG-PPS). Already known and reported metabolites from the literature were also added to the database (Hu et al., 2007; Perez et al., 2005; Trovo et al., 2009;Sirtori et al., 2010; Michael et al., 2012; Margot, 2015), if not present. For the extraction of the exact mass of the pseudomolecular ion of the suspect TPs,a data processing software was employed (Bruker Compass TargetAnalysis 1.3). In order to characterize an exact mass as a possible TP, the following preset criteria must be met: mass error ≤ 5 mDa, isotopic fit ≤ 200 mSigma, intensity threshold > 500, peak area threshold >2000, as well as absence of the ion from the control sample. Results were inspected manually, especially for the low intensity peaks not complying isotopic fit or mass accuracy criteria, decreasing the false negative results. Then, MS/MS spectra of possible TPs were examined for tentative identification and structure elucidation.

Regarding non-target screening, the initial crucial step is subtraction of each control sample from its respective treated sample, to expose masses that are exclusively detected in the treated samples. This was achieved using Bruker Compass MetaboliteDetect 2.0 software, which allows the sophisticated comparison of two full scan LC-MS data sets, creating a peak list containing exact mass and retention time (Rt) information. SmartFormula algorithm was then used to create possible sum formulaes for each exact mass, taking into account element restrictions (C, H, N, O and S), mass tolerance 5 mDa, the hydrogen to carbon ratio (H/C) from 0 to 3, check for ring and double bonds and electron configuration even for the MS and both odd and even for MS/MS peak.

6.4.2 Tables

Table S3.4.1 Chemical structures and physicochemical properties of studied antimicrobials.

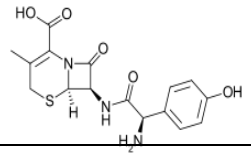
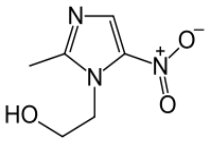
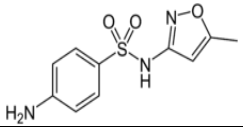
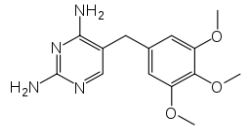
Antimicrobial	Category	Cas Number	Formula	Structure	MW[a]	pKa[b]	S_w[c]	$LogK_{ow}$[d]	$LogD_{ow}$[e]
Cefadroxil	Cephalosporin	66592-87-8	$C_{16}H_{17}N_3O_5S$		363	7	1110	-0.4	n.a
Metronidazole	Imidazole	443-48-1	$C_6H_9N_3O_3$		171	3.09	9500	-0.02	-0.01
Sulfamethoxazole	Sulphonamides	723-46-6	$C_{10}H_{11}N_3O_3S$		253	6	3942	0.89	-0.54
Trimethoprim	Bacteriostatic	738-70-5	$C_{14}H_{18}N_4O_3$		290	7.12	400	0.91	0.47
[a] MW: molecular weight; [b]pKa: dissociation constant; [c]Sw: solubility at 25°C (mg L^{-1}); [d] Log K_{ow}: octanol-water partition coefficient; [e] Log D_{ow}: octanol-water distribution coefficient at pH=7.4									
Structure and physico-chemical data source: ChemIDplus Advanced (http://chem.sis.nlm.nih.gov/chemidplus/), ChemSpider (http://www.chemspider.com/), DrugBank (http://www.drugbank.ca/drugs).									

Table S3.4.2 Experimental conditions used in batch experiments for the investigation of different mechanisms affecting target compounds removal. The duration of the experiments was 24 days, 2 gr of fresh biomass were added in flasks with *Lemna minor*, the target antimicrobials (CDX, METRO, TMP, SMX) were added in mixture.

Experiment	**Light**	**Biomass**	**Target Compounds (μg L^{-1})**	**pH**	**T (°C)**	**Replicates**	**Studied mechanism**
A	no	no	250	7.0 ± 0.2	24.0 ± 0.5˚C	3	hydrolysis
B	yes	no	250	7.0 ± 0.2	24.0 ± 0.5˚C	3	photodegradation
C	yes	yes, inactivated	250	7.0 ± 0.2	24.0 ± 0.5˚C	3	sorption
D	yes	yes, active	250	7.0 ± 0.2	24.0 ± 0.5˚C	3	plant uptake

Table S3.4.3 Experimental conditions used in batch experiments for the identification of transformation products (TPs) of target compounds. The duration of the experiments was 24 days, 2 gr of fresh biomass were added in flasks with biomass (*Lemna minor*).

Experiment	Light	Biomass	Concentration ($\mu g\ L^{-1}$)	Target Compound	pH	T (°C)	Replicates
A	yes	no	Only Medium SIS	-	7.0 ± 0.2	24.0 ± 0.5˚C	3
B1	yes	no	1000	CFD	7.0 ± 0.2	24.0 ± 0.5˚C	3
B2	yes	yes	1000	CFD	7.0 ± 0.2	24.0 ± 0.5˚C	3
C1	yes	no	1000	METRO	7.0 ± 0.2	24.0 ± 0.5˚C	3
C2	yes	yes	1000	METRO	7.0 ± 0.2	24.0 ± 0.5˚C	3
D1	yes	no	1000	TRI	7.0 ± 0.2	24.0 ± 0.5˚C	3
D2	yes	yes	1000	TRI	7.0 ± 0.2	24.0 ± 0.5˚C	3
E1	yes	no	1000	SMX	7.0 ± 0.2	24.0 ± 0.5˚C	3
E2	yes	yes	1000	SMX	7.0 ± 0.2	24.0 ± 0.5˚C	3

Table S3.4.4 Removal of selected antimicrobials in batch experiments conducted under different experimental conditions (duration 24 days)

	CFD	**METRO**	**TRI**	**SMX**
Experiments conducted under dark conditions (Experiment A)				
Removal Efficiency (%)	71± 1	11± 2	20± 2	12 ± 1
Experiments conducted in the presence of light (Experiment B)				
Removal Efficiency (%)	92± 0	25 ± 0	33 ± 1	26± 1
Experiments conducted using inactivated biomass (Experiment C)				
Removal Efficiency (%)	100± 0	34± 2	33± 1	40 ± 1
Experiments conducted using active biomass (Experiment D)				
Removal Efficiency (%)	100± 0	96± 0	59 ± 1	73± 0

Table S3.4.5 Predicted LC50 values of parent pharmaceuticals and their identified transformation products (according to Aalizadeh et al., ESPI, 2017)[a]

Compound Name	*Daphnia Magna* (48h) (g/L)	*PimephalesPromelas* (96h) (g/L)	*Pseudokirchneriellas ubcapitata* (72h) (g/L)
Trimethoprim	1074	1071	1632
Trimeth-306a	1198	1198	1682
Trimeth-306b	1238	1195	1780
Trimeth-306c	1253	1219	1722
Trimeth-304	1205	-[b]	1716
Trimeth-322a	1267	1338	1882
Trimeth-322b	1331	1283	1879
Trimeth-322c	1292	1347	2794
Trimeth-141	355	231	611
Trimeth-139	537	297	601
Trimeth-324a	944	1258	1625
Trimeth-324b	983	1297	1719
Trimeth-276a	1102	948	1467
Trimeth-276b	1113	928	1569
Trimeth-294a	912	1062	1433
Trimeth-294b	930	1051	1580
Metronidazole	4.07	3.31	3.78
Metronid-185a	3.61	3.84	3.66
Metronid-185b	3.36	3.89	4.02
Metronid-185c	3.53	3.85	3.95
Metronid-185d	3.87	3.82	4.05

[a] Reza Aalizadeh, Peter C. von der Oheand Nikolaos S. Thomaidis"Prediction of Acute Toxicity of Emerging Contaminants on the Water Flea Daphnia magna by Ant Colony Optimization - Support Vector Machine QSTR models" Environmental Science: Processes & Impacts, under revision, 2016 (invited article in the special

issue: QSARs and computational chemistry methods in environmental chemical sciences)

[b] Not estimated; outside of the applicability domain of the model.

Table S3.4.6 Chemical characteristics of wastewater at different points of duckweed *Leman minor*continuous-flow system (for pH, T and conductivity measurements, n = 78; for DO measurements, n = 28)

	pH	**T (˚C)**	**DO (mgL^{-1})**	**Conductivity ($\mu S\ ms^{-1}$)**
Influent wastewater	8.01 ± 0.15	23.3 ± 0.3	-	1191 ± 115
Pond 1	8.38 ± 0.24	22.0 ± 1.7	5.6 ± 1.7	1313 ± 92
Pond 2	8.76 ± 0.30	22.2 ± 1.4	7.2 ± 2.4	1380 ± 113
Pond 3	8.81 ± 0.35	22.4 ± 1.5	6.6 ± 2.7	1518 ± 189

Table S3.4.7 Removal of conventional pollutants in continuous-flow system with *Lemna minor* (for COD, NH_4-N and NO_3-N measurements, n = 18; for TP measurements, n = 5).

	COD ($mg\ L^{-1}$)	**NH_4 – N ($mg\ L^{-1}$)**	**NO_3 – N ($mg\ L^{-1}$)**	**TP ($mg\ L^{-1}$)**
Inlet – Point A	114± 10	3.8 ± 0.3	1.7 ± 0.5	5.3 ± 0.4
Outlet – Point D	45 ± 7	0.2 ± 0.2	1.3 ± 0.4	1.0 ± 0.2
Removal %	*61 ± 5*	*96± 1*	*28 ± 2*	*81 ± 2*

Table S3.4.8 Concentrations of target antimicrobials in influent wastewater and in the effluents of the different ponds consisting the continuous-flow *Lemna minor* system (n = 7). The performance of each pond has been calculated using as C_o the concentrations of target compounds in influent wastewater of the system (total removal).

	Point A (Inlet)	**Point B (Outlet of Pond 1)**	**Point C (Outlet of Pond 2)**	**Point D (Outlet of Pond 3)**
METRO (μg L^{-1})	7.8 ± 0.2	6.0 ± 1.8	3.7 ± 1.8	2.4 ± 0.8
METRO total removal (%)		*25 ± 20*	*49 ± 25*	*71 ± 11*
TRI (μg L^{-1})	14.3 ± 4.7	10.7 ± 4.7	6.7 ± 4.3	5.4 ± 0.9
TRI total removal (%)		*30 ± 20*	*54 ± 14*	*61 ± 8*

6.4.3 Figures

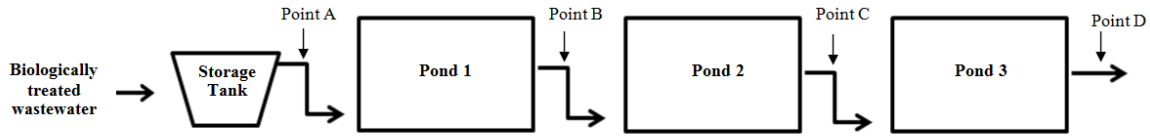


Figure S3.4.1 Schematic diagram of the continuous flow lab-scale system used in this study. The sampling points for wastewater (Point A: inlet; Point B: outlet of 1st Pond; Point C: outlet of 2nd Pond; Point D: outlet of 3rd Pond) are also presented.

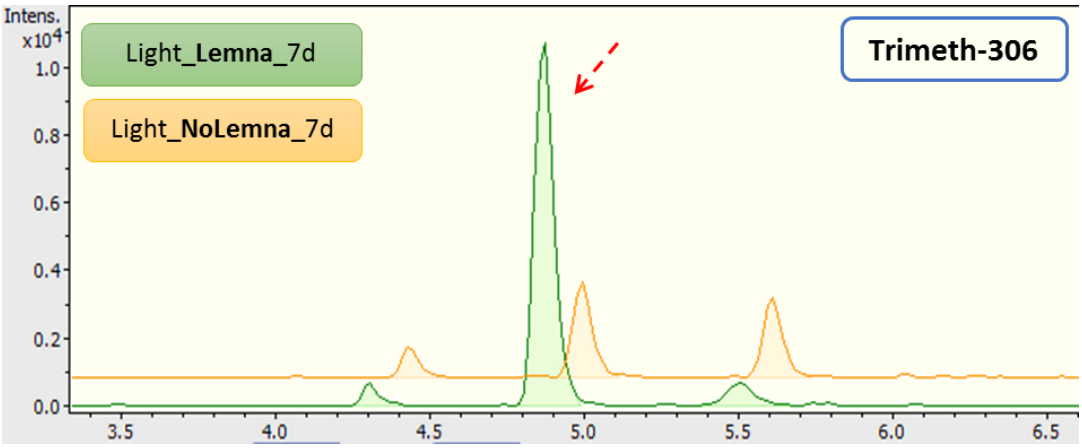


Figure S3.4.2 Extracted ion chromatogram of the suspect transformation product Trimeth-306 in the presence and absence of *Lemna minor*.

6.4.4 References

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