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“Removal of bisphenol A from wastewater and groundwater with helophytes”

«Απομάκρυνση δισφαινόλης Α από λύματα και υπόγειο νερό με χρήση ελόφυτων»

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This thesis is dedicated to
Konstantinos, Marios,
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List of acronyms

Abbreviation	Meaning
ACN	Acetonitrile
BaP	Benzo(a)pyrene
BOD	Biochemical Oxygen Demand
BPA	Bisphenol A
BTEX	Benzene, oluene, ethylbenzene, and xylene
CAFOs	Concentrated animal feeding operations
CAT	Catalase
CEC	Contaminants of Emerging Concern (or EOCs)
CIP	Ciprofloxacin
COD	Chemical Oxygen Demand
CW(s)	Constructed wetland(s) (syn.: artificial or engineered wetlands)
DAD	Diode Array Detector
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
dw	Dry weight
EC	Electrical Conductivity
EDTA	Ethylenediaminetetraacetic acid
EEC	European Economic Community
EFSA	European Food Safety Authority
ENR	Enrofloxacin
EOCs	Emerging Organic Contaminants (or CEC)
EPA	United States Environmental Protection Agency (or USEPA)
ET	Evapotranspiration
FLD	Fluorescence Detector
FQs	Fluoroquinolones
FWS	Free Water Surface (or open water)
GC	Gas Chromatography
GPX	Guaiacol Peroxidase
HM	Heavy Metals

HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention (or Residence) Time
HSF	Horizontal Subsurface Flow
IC	Inorganic Carbon
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
MBR	Membrane Bioreactor
PC	Polycarbonate
PCBs	Polychlorinated biphenyls
PEB	Polyethylene bags (experiment)
PPCPs	Pharmaceuticals and Personal Care Products
PVC	Polyvinyl chloride
S.E.	Standard Error
SS	Suspended Solids
SSF	Subsurface Flow System
SMX	Sulfamethoxazole
TC	Total Carbon
TET	Tetracycline
TN	Total Nitrogen
TOC	Total Organic Carbon
TP	Total Phosphorus
TSS	Total Suspended Solids
TUC	Technical University of Crete
USFDA	U.S. Food and Drug Administration
UV	Ultraviolet
UV-VIS	Ultraviolet–Visible Spectroscopy
VF	Vertical flow
VFS	Vertical Flow Systems
WW	Wastewater
WWTP(s)	Wastewater Treatment Plant(s)

ABSTRACT

The presence of emerging organic contaminants (EOCs) in wastewater (WW) has attracted intense interest in the environmental engineering community, due to the high frequency of detection and the adverse effects on human health and the environment. EOCs consist of a wide range of substances, such as endocrine disrupting compounds (EDCs), pesticides and pharmaceuticals, such as antibiotics. EDCs are exogenous substances, which even in very small amount interact with the hormonal regulation and the endocrine system. On the other hand, the presence of antibiotics in the environment has raised increasing awareness, because they can potentially cause the selective proliferation of antibiotic resistant bacteria.

Conventional WW treatment plants (WWTPs) have proven to be inefficient in the removal of EOCs, since they are not currently designed to cope with this type of contamination. Taking into consideration that nowadays WW discharges still constitute one of the major sources of EOCs in the environment, addressing this threat has become a top-priority task. Implementation of efficient advanced tertiary treatment techniques, are currently very energy- and cost-intensive technologies. Among alternative treatment technologies, constructed wetlands (CWs) are proposed as an environmentally acceptable method that exploits the synergistic effect of plants with the associated microorganisms for WW treatment, contaminated with EOCs. Current knowledge on the efficacy of the removal of EOCs with CWs and the effect of various parameters, is still limited.

In this regard, the objective of this thesis is to investigate the potential of specific halophytes to remove the endocrine disruptor BPA from municipal wastewater and groundwater. Moreover, to evaluate the performance of two CWs planted with halophytes on the removal of selected EOCs. We focused on the contribution of plants on the removal, as well as the efficacy of the planted wetland as a whole system.

Pot-scale experiments were performed, in order to have a first view of the role of *Juncus acutus* and *Tamarix parviflora* halophytes on BPA removal from contaminated soil. Estimation of the apparent degradation rate constant (*k'*) revealed the contribution of halophytes to the process. Degradation rates was found to be 1.94-2.47 10^{-2} in the planted and 0.71-0.98 10^{-2} mg BPA kg^{-1} soil h^{-1} in non-planted treatments.

Following this, a hydroponic experiment took place with the helophyte *J. acutus* be exposed to mixture of contaminants: BPA, antibiotics ciprofloxacin (CIP) and sulfamethoxazole (SMX) and heavy metals (Cr, Cd, Zn and Ni). The aim was to evaluate the potential of *J. acutus* for use in CWs. Concentrations of the compounds ranged from μg L^{-1} to well beyond

environmentally relevant values (50 mg L^{-1} for the organics and >1000 mg L^{-1} for Zn and Cr). *J. acutus* exhibited significant contribution on the removal of BPA, CIP and SMX. The resultant removal of BPA concentrations of up to 50 mg L^{-1} exceed 96%, after 28 days. *J. acutus* plants show the potential to accumulate heavy metals in both root and leaf tissues in considerable amounts.

The aim of the next study was to simulate the treatment procedure of a contaminated shallow aquifer. A pilot unit of 1 m^3 planted with *J. acutus* used spiked with BPA tap water that was recirculated, flowing from the bottom to the surface of the pilot, passing through the root zone. When the system exposed to initial BPA concentrations of 2.67 mg L^{-1} and 184 μg L^{-1} could not be detected after 15 days of treatment. After 5 experimental runs, the system was proved reliable and robust within reasonable input oscillations of the BPA concentration and inlet flow rate.

Afterwards, evaluation of a small scale horizontal subsurface flow (HSF) CW fed with secondary treated WW from the WWTP of the city of Chania, was performed. The HSF of 0.5 m^3 surface and 157 L working volume was planted with 5 *Juncus acutus* helophytes. The aim was to investigate system's efficiency in terms of BPA and antibiotics removal, in short term experiments (approx. 14 days) and under different operating conditions. The influence of vegetation on BPA removal was highlighted after comparison with a non-vegetated unit. The ratio of BPA mass removed over the BPA mass entering the system (m_p%) was 46% higher in the vegetated wetland. Superior treatment's performance at hydraulic retention time (HRT) of 2 d was obtained: m_p was 92% versus 48% at HRT of 1 d. The influence of HRT on CIP removal was less important. SMX was resistant to the removal and demonstrated unstable results. A slightly higher BPA removal efficiency in summer was not statistically verified.

Finally, a pilot-scale HSF-CW study for the degradation of bisphenol A in primary-treated municipal wastewater, was undertaken for 7 months period. The average removal efficiency for BPA was 98% (mean influent concentration: 0.26 mg L^{-1}) in the summer. Significant sensitivity in the system was observed with changes of HRT. For almost all the wastewater quality parameters, moderate removal efficiencies were measured.

The overall outcome of this study is the significant contribution of *Juncus acutus* to the attenuation of BPA. It appears as a promising species for CW applications and is recommended for further investigation in the phytoremediation field.

ΠΕΡΙΛΗΨΗ

Η παρουσία των αναδυόμενων οργανικών ρύπων (EOCs) σε αστικά λύματα (WW) έχει προκαλέσει το ενδιαφέρον της επιστημονικής κοινότητας, λόγω της συχνότητας προσδιορισμού τους και τις επιπτώσεις που δύναται να έχουν στην ανθρώπινη υγεία και στο περιβάλλον. Οι EOCs περιλαμβάνουν ένα ευρύ φάσμα ενώσεων, στις οποίες ανήκουν οι ενδοκρινικοί διαταράκτες (EDCs), τα παρασιτοκτόνα και οι φαρμακευτικές ενώσεις, όπως τα αντιβιοτικά. Οι EDCs είναι εξωγενείς ουσίες οι οποίες ακόμα και σε πολύ μικρή συγκέντρωση επιδρούν στο ορμονικό και ενδοκρινικό σύστημα των οργανισμών. Από την άλλη πλευρά, η παρουσία των αντιβιοτικών στο περιβάλλον έχει επιστήσει την προσοχή, επειδή δυνητικά μπορεί να προκαλέσει επιλεκτικό πολλαπλασιασμό βακτηρίων ανθεκτικών στα αντιβιοτικά.

Οι συμβατικές εγκαταστάσεις επεξεργασίας λυμάτων (ΕΕΛ) έχουν αποδειχθεί ανεπαρκείς για την απομάκρυνση των EOCs, δεδομένου ότι δεν έχουν σχεδιαστεί για την επεξεργασία αυτής της κατηγορίας ρύπων. Λαμβάνοντας υπόψη ότι οι εκροές των λυμάτων εξακολουθούν να αποτελούν μία από τις κυριότερες πηγές εισόδου των EOCs στο υδάτινο περιβάλλον, η μείωση της επικινδυνότητας των αποβλήτων αποτελεί στόχο υψηλής προτεραιότητας. Μεταξύ των εναλλακτικών τεχνολογιών επεξεργασίας, οι τεχνητοί υγροβιότοποι (ΤΥ) προτείνονται ως μια περιβαλλοντικά αποδεκτή μέθοδος η οποία εκμεταλλεύεται την συνεργιστική δράση των φυτών και τους σχετιζόμενους με αυτά μικροοργανισμούς, για την επεξεργασία λυμάτων ρυπασμένων με EOCs. Οι γνώσεις σχετικά με την αποτελεσματικότητα της απομάκρυνσης των EOCs με ΤΥ και η επίδραση διαφόρων παραμέτρων στην απομάκρυνσή τους, είναι ακόμα περιορισμένη.

Υπό αυτό το πρίσμα, ο στόχος της διατριβής αυτής είναι να μελετηθεί η συμβολή επιλεγμένων ελόφυτων στην απομάκρυνση του ενδοκρινικού διαταράκτη δισφαινόλη Α (BPA) από αστικά λύματα και υπόγεια ύδατα. Στο πλαίσιο αυτό αξιολογήθηκε η απόδοση της λειτουργίας δύο ΤΥ όσον αφορά στην απομάκρυνση επιλεγμένων EOCs. Πιο συγκεκριμένα, εστιάσαμε αφενός στο ρόλο των φυτών στην απομάκρυνση αυτή και αφετέρου στην αποτελεσματικότητα του ΤΥ ως σύστημα εξυγίανσης.

Αρχικά διεξήχθησαν πειράματα μικρής κλίμακας ώστε να γίνει μια πρώτη εκτίμηση της συμβολής του *Juncus acutus* (βούρλο) και του *Tamarix parviflora* (αρμυρίκι) στην απομάκρυνση της BPA από ρυπασμένο έδαφος. Ο υπολογισμός του φαινομενικού ρυθμού απομάκρυνσης (*k'*), κατέδειξε την συνεισφορά των φυτών στην απομάκρυνση. Οι ρυθμοί απομάκρυνσης βρέθηκαν 1.94-2.47 10^{-2} παρουσία φυτών και 0.71-0.98 10^{-2} mg BPA kg^{-1} soil h^{-1} απουσία φυτών.

Με βάση τα αποτελέσματα, σχεδιάστηκε ένα υδροπονικό πείραμα κατά το οποίο υδρόβια φυτά, *J. acutus,* εκτέθηκαν σε μικτή ρύπανση με BPA, τις αντιβιοτικές ουσίες σιπροφλοξασίνη (CIP) και σουλφαμεθοξαζόλη (SMX) και βαρέα μέταλλα (χρώμιο, ψευδάργυρο, νικέλιο και κάδμιο). Με αυτόν τον τρόπο εξετάστηκε η απόκριση των φυτών για χρήση σε σύστημα ΤΥ. Οι προστιθέμενες συγκεντρώσεις κυμάνθηκαν από επίπεδα μg L^{-1} έως και >1000 mgL^{-1} (για Zn και Cr). Το *J. acutus* συνέβαλε σημαντικά στην απομάκρυνση συγκεντρώσεων της BPA έως και 50 mg L^{-1} σε ποσοστό άνω του 96%, σε διάστημα 28 ημερών. Επίσης, τα ελόφυτα συσσώρευσαν σημαντικές ποσότητες βαρέων μετάλλων στους ιστούς της ρίζας και των φύλλων.

Στη συνέχεια της μελέτης έγινε προσομοίωση επεξεργασίας ρηχού υδροφορέα παρουσία ελόφυτων με εκτίμηση των παραμέτρων που επιδρούν στην απομάκρυνση των ρύπων. Για το σκοπό αυτό χρησιμοποιήθηκε μία πιλοτική μονάδα, όγκου 1 m^3, με *J. acutus*, στην οποία γινόταν επανακυκλοφορία του ρυπασμένου με BPA νερού, από τον πυθμένα προς την επιφάνεια της μονάδας, περνώντας μέσα από τη ριζόσφαιρα. Όταν το σύστημα εκτέθηκε σε συγκεντρώσεις BPA 2.67 mg L^{-1}-184 μg L^{-1}, παρατηρήθηκε απομάκρυνση της ένωσης κάτω από τα όρια ανίχνευσης μετά από 15 ημέρες. Το σύστημα υποβλήθηκε σε πειραματικούς κύκλους ώστε να επιβεβαιωθεί η αξιοπιστία και η ευρωστία του σε πραγματικές πιέσεις, με βάση εύλογες διαταραχές τόσο της συγκέντρωσης ρύπων στην εισροή, όσο και της ογκομετρικής παροχής.

Ακολούθως, εξετάστηκε ένας ΤΥ οριζόντιας υποεπιφανειακής ροής, ο οποίος τροφοδοτούταν από δευτεροβάθμια επεξεργασμένη εκροή αστικών λυμάτων, στην ΕΕΛ Χανίων. Ο ΤΥ είχε επιφάνεια 0.5 m^2, λειτουργικό όγκο 157 L και ήταν φυτεμένος *J. acutus.* Ο σκοπός ήταν η διερεύνηση της αποτελεσματικότητας του συστήματος στην απομάκρυνση των BPA, CIP και SMX κάτω από διαφορετικές συνθήκες λειτουργίας, σε πειραματικούς κύκλους 14 ημερών. Υπό αυτές τις συνθήκες, αξιολογήθηκε η δυναμική της βλάστησης στην απομάκρυνση της BPA μέσω σύγκρισης με αντίστοιχο μη φυτεμένο ΤΥ ελέγχου. Ο λόγος της απομακρυνόμενης μάζας της BPA προς αυτή που εισάγεται στο σύστημα (m_p%) βρέθηκε κατά 46% υψηλότερος στον φυτεμένο υγροβιότοπο. Η μεταβολή του χρόνου παραμονής (HRT) προκάλεσε σημαντική διαφοροποίηση στην απομάκρυνση της BPA. Ο λόγος m_p βρέθηκε ίσος με 92% σε HRT 2 d και 48% σε HRT 1 d. Αντίστοιχα, η επίδραση του HRT στην απομάκρυνση της CIP ήταν μικρότερη. Από την άλλη, η SMX αποδείχθηκε ανθεκτική στην επεξεργασία και έδειξε ασταθή αποτελέσματα αναφορικά με τη δυνατότητα απομάκρυνσής της. Επίσης, διαπιστώθηκε μικρή αύξηση του ποσοστού απομάκρυνσης της BPA κατά τη θερμή περίοδο, χωρίς να επιβεβαιωθεί στατιστικά.

Τέλος, πραγματοποιήθηκε μια έρευνα με ΤΥ πιλοτικής κλίμακας διάρκειας 7 μηνών για την απομάκρυνση της BPA από αστικά λύματα πρωτοβάθμιας επεξεργασίας. Η μέση απομάκρυνση των 0.26 mg L^{-1} BPA ήταν 98%. Σημαντική ευαισθησία του συστήματος ως προς την απομάκρυνση της BPA παρατηρήθηκε με τη μεταβολή του HRT. Η απόδοση ως προς τις φυσικοχημικές παραμέτρους ήταν μέτρια.

Η μεθοδολογία προσέγγισης του αντικειμένου, ο πειραματικός σχεδιασμός και κυρίως η συνεισφορά του *J. acutus* στην απομάκρυνση αναδυόμενων οργανικών ρύπων, στην παρούσα διατριβή, αποτέλεσε σημαντικό βήμα για την εκτίμηση της αποτελεσματικότητάς του σε συστήματα ΤΥ. Συμπερασματικά η εφαρμογή του συγκεκριμένου είδους ελόφυτου συνιστάται για περαιτέρω διερεύνηση στον τομέα της φυτοεξυγίανσης.

CHAPTER 1. INTRODUCTION AND AIMS

Water pollution is an issue of major concern worldwide. Urbanization and industrialization that radically increased the release of anthropogenic compounds to the environment, through untreated or partially treated municipal and industrial wastewater to the receivers (Ávila et al., 2013). Emerging organic contaminants (EOCs) consist of a large group of synthetic and natural compounds, commonly detected in municipal, industrial, hospital or agricultural wastewater (Talib and Randhir, 2016). This group includes pesticides, flame retardants, pharmaceuticals and personal care products (PPCPs), endocrine disrupting compounds (EDCs) and other pollutants (Ahmed et al., 2016; Kuster et al., 2008). Known also as Contaminants of Emerging Concern (CEC), they have the potential to cause adverse effects to the environment, human health or living organisms even at low levels (Noguera-Oviedo and Aga, 2016).

Apart from inefficient wastewater treatment, other important sources of EOCs are household and industrial waste, runoff, agricultural activities (crops and livestock excreta), hospital effluents, aquaculture discharges and wastes of pharmaceutical manufacturing companies (Lapworth et al., 2012; Pal et al., 2010; Stuart et al., 2012; Talib and Randhir, 2016). EDCs, such as pesticides, biocides, chemical catalysts or plasticizers, may influence reproductively-relevant or nonreproductive, sexually-dimorphic behaviors, or under certain circumstances may result in more salient effects on neurodevelopmental and⁄or reproductive processes (Frye et al., 2012). On the other hand, antibiotics, a specific group of PPCPs, is a growing concern due to the fact that long-term exposure in low concentrations can promote the spread of antibiotic resistant bacteria (Ahmed et al., 2016; Bhandari et al., 2008; Sui et al., 2015). Consequently, resistant genes may be transferred to human pathogens, creating a high-risk potential for human health (Pal et al., 2014).

The removal of the above contaminants has been attracting the attention of scientific and industrial community the last decades, with variable treatment efficiencies (Ahmed et al., 2016; Sui et al., 2015). Different technologies have been used for this aim; biological (bioreactors of various types, activated sludge, nitrification etc.), chemical (coagulation, ozonation, photocatalysis, Fenton and foto-Fenton etc.) or physical (reverse osmosis, sedimentation, nanofiltration, micro- or ultrafiltration etc.). Biological technologies are primarily based on biodegradation, being the most popular among them (Ahmed et al., 2016). In this regard, advanced tertiary treatment technologies, adapted recently in WWTPs, revealed up to 99% removal of specific EOCs. However, high cost and energy consumption are major drawbacks that limit their application (Garcia-Rodríguez et al., 2014).

Constructed wetlands (CW), first tested in the early 1950s (Tore et al., 2012) are classified as a non-conventional biological treatment technology (Ahmed et al., 2016). These low cost, simple and environmental friendly artificial systems, take advantage of the biological, physical and chemical processes occurring in natural wetlands, using plants and their associated microorganisms, for wastewater treatment (Zhang et al., 2015). In recent years there has been considerable interest in the contribution of treatment wetlands to the attenuation of EOCs using artificial wetlands (Garcia-Rodríguez et al., 2014). Of special interest in this field is the investigation of removal mechanisms with emphasis on the effect of different parameters such as vegetation, artificial aeration, the support matrix and environmental conditions (Dordio and Carvalho, 2013; Li et al., 2014a). Nonetheless, current knowledge is limited and there is still considerable ambiguity with regard to the removal efficiencies, the mechanisms involved and the influence of certain parameters (Li et al., 2014b).

Studies investigating the role of macrophyte species on the efficiency of CWs, agreed that selection of plant does matter (Brisson and Chazarenc, 2009). Although CWs is not a new developed technology, wetland species tested for this purpose are limited. In the key study undertaken by Stottmeister et al. (2003) the synergistic effects of plants and their associated microorganisms is being assessed, and the processes on the root zone, the gas transport and uptake mechanisms are extensively discussed. Recently, Syranidou et al. (2016) demonstrated the potential of *J. acutus* endophytic bacteria to remove bisphenol A (BPA) from soil. Several strains were found not only tolerant to BPA, but even to be using BPA, ciprofloxacin (CIP) and sulfamethoxazole (SMX) as their sole carbon source. In this regard, Shelef et al. (2013) elaborated on the role of plants in CWs, especially pointing out the contribution of halophytes to salt remediation and their role as water quality phytoindicators. As a general conclusion, authors agree that research on plant selection for specific remediation applications is still needed. What is more, the consequent need of more rigorous experiments performed under a variety of conditions becomes of high importance.

In this aspect, the aim of this study was to investigate the potential of specific halophytes to remove the endocrine disruptor BPA from municipal wastewater and groundwater. Additionally, to evaluate the efficiency of two CWs planted with halophytes on the removal of selected EOCs. Of special interest, was a meso-scale horizontal flow constructed wetland planted with *Juncus acutus*, dealing with the removal of BPA and the antibiotics ciprofloxacin (CIP) and sulfamethoxazole (SMX). These antibiotics were chosen due to their frequency of detection in wastewater and other multiple aquatic environments, as well as their relatively high concentrations detected (Ávila et al., 2010; Sui et al., 2015). We attempted focusing on the individual contribution of plants on the removal and the efficacy of the planted wetland as a whole system.

For this reason, a set of different scale experiments was designed. The initial small-scale experiments assisted to gain a first evaluation of halophytic vegetation to BPA removal. These were performed with the halophytes *Juncus acutus* and *Tamarix parviflora* growing on BPA-contaminated soil. From this study, a first evaluation of the impact of vegetation on BPA removal is attempted, as well as a comparison between plant species, through the estimation of apparent first order degradation rate constant. Subsequently, a hydroponic experiment was carried out to investigate the contribution of *J. acutus* on the removal of a mixture of contaminants: BPA, antibiotics and heavy metals (H.M.) in different concentrations. Seven treatments at each replicate aimed at increasing the precision of statistical tests. Afterwards, a mesocosm study was carried out, using a rhizodegradation pilot unit of 1 m^3 and planted with two *J. acutus* plants. Experimental design aimed to simulate treatment procedure of a contaminated shallow aquifer. The system was operated and evaluated for its capacity on BPA removal, at different hydraulic retention times (HRTs). Moreover, its robustness was evaluated through a substantially increase of the influent concentration.

The final purpose of this study was to test the selected halophyte, on real conditions. Therefore, two horizontal subsurface flow (HSF) CWs were tested for their efficiency on organic pollutants' removal from municipal wastewater. The HSF-CW of 1 m^2 surface, planted with five *Juncus acutus* halophytes, was specifically designed and constructed for the purposes of this study. It was operated in the WWTP of the city of Chania and fed with secondary treated wastewater, spiked with BPA, CIP and SMX. Important operating parameters such as the impact of vegetation, HRT and seasonality were tested and an overall evaluation of the system's efficiency was attempted. The last experiment dealt with primary treated municipal wastewater with a larger scale CW. The system consisted of a surface flow (SF) CW and a HSF-CW of 45.36 m^2, in series. BPA was spiked to the wastewater and removal of the compound was monitored, at different HRT for a period of seven months.

We aware that in this research no further analysis on the contaminants removal mechanisms, such as the uptake into plant tissue, is included. Some of these analyses were performed, but without the desirable outcomes. However, a comprehensive literature review on this issue was carried out.

In this thesis, apart from the analysis of the organic contaminants, further parameters were monitored, such as water quality characteristics, nutrients, evapotranspiration, heavy metals uptake in plant tissues and physiological parameters of plants. Moreover, for the needs of this study, a new method for the simultaneous determination of CIP and SMX was developed and successfully applied.

CHAPTER 2. THEORETICAL BACKGROUND

2.1. Emerging Organic Contaminants

Emerging organic contaminants (EOCs) are complex synthetic or naturally occurring molecules or even any micro-organism, that are not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects (Kuster et al., 2008). Even though EOCs are not relatively new pollutants, only in the last 20 years they have been classified and widely investigated due to the development of sensitive analytical methods. EOCs concentration in environmental samples are significantly low and may range from a few ng L^{-1} to a few hundred μg L^{-1} (Noguera-Oviedo and Aga, 2016; Ahmed et al., 2016).

EOCs include anthropogenic and naturally occurring chemicals such as pharmaceuticals and personal care products (PPCPs), metabolites and transformation products of PPCPs and illicit drugs (Lapworth et al., 2012). Major sources of these pollutants are the discharge of wastewater, with incompletely removed organic contaminants into the surface water (Kolpin et al., 2004), hospital wastes (biohazardous materials and expired drugs), animal operations wastes (hormones and drugs injected to poultry and cattle), aquaculture discharges (hormones fed to fish; antibiotics added to feed and waters) crop and livestock operations, operational byproducts, household discharges (expired and consumed drugs from leaky sewers and septic systems), wastes of drug manufacturing companies (industrial wastes containing drugs; storm runoffs carrying powdered drugs) and from WWTP (residuals from wastes and sewage containing drugs and hormones).

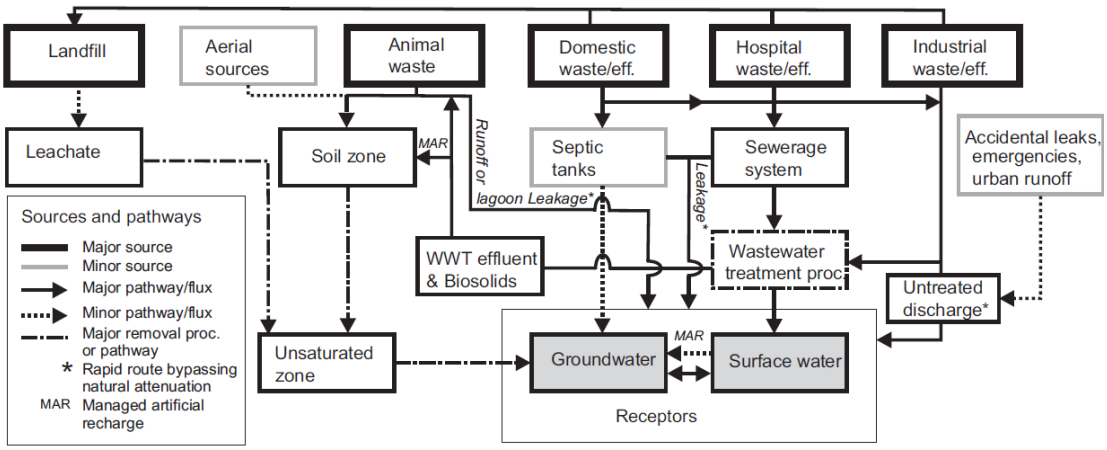


Figure 1. Route of EOCs from major sources to groundwater and surface water (adapted by Lapworth et al., 2012).

Table 1 summarizes the main classes of EOCs by their primary sources. Using the source-pathway-receptor approach, the route for EOCs from each source, is presented schematically in Fig. 1.

Table 1. Main classes of EOCs by their primary sources (data adapted by Luo et al., 2014).

Category	Important subclasses	Major sources	
		Distinct	Nonexclusive
Pharmaceuticals	NSAIDs, lipid regulators, anticonvulsants, antibiotics, β-blockers, and stimulants	Domestic wastewater, hospital effluents, run-off from CAFOs and aquaculture	Sources that are not exclusive to individual categories include: Industrial wastewater (from product manufacturing discharges) Landfill leachate (from improper disposal of used, defective or expired items)
Personal Care Products	Fragrances, disinfectants, UV filters, and insect repellents	Domestic wastewater (from bathing, shaving, spraying, swimming and etc.)	
Steroid hormones	Estrogens	Domestic wastewater (from excretion) Run-off from CAFOs and aquaculture	
Surfactants	Non-ionic surfactants	Domestic wastewater (from bathing, laundry, dishwashing and etc.) Industrial wastewater (from industrial cleaning discharges)	
Industrial chemicals	Plasticizers, fire retardants	Domestic wastewater (by leaching out of the material)	
Pesticides	Insecticides, herbicides and fungicides	Domestic wastewater (from improper cleaning, run-off from gardens, lawns and roadways etc.) Agricultural runoff	

*CAFOs: Concentrated animal feeding operations.

A lot of information already exists on the regulated of contaminants with respect to human health, but there is little knowledge about the influence of the EOCs, which have the potential to cause known or suspected adverse ecological (drinking water supplies, ecosystem) or human health effects.

Furthermore, bioaccumulation of EOCs adversely impacts and organism's hormonal system, provoking abnormality and consequently causing reproductive impairments, decreased fecundity, increased incidence of breast and testosterone cancers, and persistent antibiotic resistance (Pal et al., 2010). Of particular concern are antibiotic residues, which can induce the development of antibiotic resistant genes potentially favoring superbugs (Ahmed et al., 2016).

2.1.1. Endocrine Disrupting Chemicals (EDCs)

Endocrine Disrupting Chemicals (EDCs) are exogenous compounds, which even in very small amounts interact with the hormonal regulation and the endocrine system (stimulating, repressing or blocking function) disrupting an animal's or human's normal homeostasis. EDCs have the ability to inhibit hormone's release and production affecting negatively endocrine systems and induce metabolic problems or promote developments of clinical disorders in humans (Toro-Velez et al., 2016). Egg breakage of birds, fishes and turtles, problems in reproductive systems, drastic changes in the immune system of marine mammals, weak male reproductive performance, increase in breast, testicular and prostate cancers and endometriosis are only some of the problems associated with EDCs (Ahmed et al., 2016).

According to literature EDCs can be classified into two major categories depending on whether the origin of the compound is natural or synthetic (Diamanti-Kandarakis et al., 2009). The first category includes natural compounds, such as phytoestrogens, genistein and coumestrol, are found in animal and human foods. The second category includes synthetic chemicals used as industrial solvents or oils and byproducts thereof. Here are compounds such as polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), dioxins bisphenol A (BPA), plasticizers, pesticides (dichlorodiphenyltrichloroethane (DDT)), fungicides, and various drugs (diethylstilbestrol (DES) (Kabir et al., 2015).

Kabir et al., (2015) reported a third group of EDCs, associated with the sources of origin. This category includes natural and synthetic hormones such as phytoestrogens and omega-3 fatty acids, pharmaceutical substances with hormonal effects (naproxen, metoprolol), chemical compounds of industrial or domestic origin (PCBs, flame retardants) and products of industrial and domestic processes (polycyclic aromatic hydrocarbons (PAHs), dioxins and pentachlorobenzene).

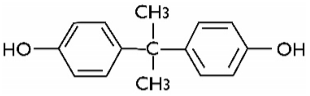
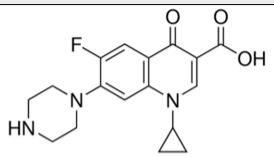
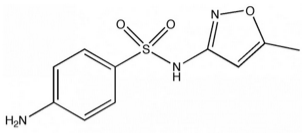
2.1.2. Bisphenol A

Physicochemical properties, production and uses

Bisphenol-A (BPA) (4,4′- isopropylidenediphenol alternatively, 2,2′-bis(4-hydroxyphenyl) propane or 2,2-(4,4′-dihydrox-ydiphenyl) propane, with molecular formula:

$(CH_3)_2C(C_6H_4OH)_2$) is an organic compound containing two hydroxyphenyl groups and belongs to the category of endocrine disruptors. BPA's octanol-water partition coefficient (log K_{ow}) is equal to 3.4, indicating high level of solubility in fats and low level in water (Syranidou, 2016). Low value of log K_{ow} apart from the lipophilicity of BPA also reveals its tendency to bind to solid phases in aquatic ecosystems (Mathieu-Denoncourt et al., 2016). BPA is an important synthetic xenoestrogen and among common environmental estrogens is the most detected EDC in groundwater samples (Lapworth et al., 2012; Peng et al., 2014).

Table 2. Chemical structure and physicochemical properties of BPA, CIP and SMX. (Papaevangelou et al., 2016; Yan et al., 2015).

Compound	Bisphenol A	Ciprofloxacin	Sulfamethoxazole
Chemical structure			
Molecular weight (g mol^{-1})	228.29	331.34	253.28
Water solubility	120-300 (g L^{-1})	30 (g L^{-1})	281 mg L^{-1} (pH = 3.22) 17,900 mg L^{-1} (pH = 7.5)
log Kow	3.4	0.28	0.89
pKa	9.6	5.90 and 8.89 6.1– 8.7	5.6

Bisphenol-A was designed in the 1890s and was used as an artificial estrogen in the early of 1930s, due to the positive action of the reproductive system of female mice in lab experiments (Giulivo et al., 2016). Nowadays, BPA is one of the most commonly produced and used chemicals in the world (Michałowicz, 2014), since is primarily used as an intermediate in the production of polycarbonate (PC) plastics, epoxy resins, phenol resins, polyesters, along with other materials widely used in the plastics industry (Goodson et al., 2002). About 65% of BPA's production is used in the plastics industry for the production of polycarbonate and 28% for epoxy resins. The residue percentage (7%) refers to other products such as fire retardants, unsaturated polyester styrene resins, polyvinyl chloride (PVC) products, etc. (Cousins et al., 2002). The produced plastic containing BPA, is used in bags, food and beverage packaging, baby bottles, metal cans, construction materials (Pal et al., 2014), dental resins of inner cover cans, medical neonatal intensive care units appliances, soap, lotion, shampoo and sunscreen (Giulivo et al., 2016). In addition, it is used as an additive in thermal paper (Mendum et al., 2011) and CDs (Deblonde et al., 2011). The global production capacity of BPA is about 4 million tons annually (Deblonde et al., 2011), while among the ranked top five producers

worldwide is the USA, Taiwan, South Korea, China and Japan according the World Market Outlook.

Environmental fate and main sources of exposure to BPA

In the past years, diffusion of BPA in the environment took place in diverse ways, since European, Japan and North American use lots of materials containing BPA. This increased the industrial BPA emissions. The treated sewage water, sludge and landfill leachate have been increasingly and continuously used for soil irrigation and amendment, so BPA is ubiquitous in soil (Flint et al., 2012). Another source of contribution to the total BPA exposure is by thermal paper through dermal contact (Corrales et al., 2015; EFSA, 2014; Geens et al., 2012). BPA exposure through environmental contamination may also occur during production, processing, use or via physical and chemical degradation of end products during disposal or recycling of substances containing BPA (Corrales et al., 2015; Møller and Larsen, 2012). Recent research has shown that man is exposed to BPA through packaged food and beverages (Aghajanpour-Mir et al., 2016; Aschberger and Castello, 2010; Chouhan et al., 2014; Corrales et al., 2015; EFSA, 2014; Flint et al., 2012; Hunt et al., 2009; Schierow and A.Lister, 2010).

Regarding the exposure to BPA, studies have reported a variety of effects on human health associated with chronic diseases such as diabetes, obesity, cardiovascular disease as well as congenital defects and disorders in the reproductive system (Syranidou, 2016). Furthermore, BPA is capable of binding to DNA via metabolic activation, has estrogenic activity at low concentrations and also has the potential to pose an increase in abnormality (Aghajanpour-Mir et al., 2016). Infants and toddlers exposed to BPA through breast milk and canned food. It has been calculated that for babies the hazard ratio (Hazard Index, HI) for exposure to BPA has to be less than one (0.002-0.26) and for adults within the range of 0.002-0.03. Although these values are low, the risk of carcinogenic diseases in humans remains (Pal et al., 2014). The US Food and Drug Administration of the United States banned in 2013 the usage of BPA in production of epoxy resins and food packages (Baluka and Rumbeiha, 2016). Also, in September 2010, Canada became the first country which declared BPA as a toxic substance (Ii and Mars, 2012). According to the European Commission, the excretion of BPA in aquatic environments was reported to be 92% of the total emitted contaminants (Deblonde et al., 2011). Furthermore, bioaccumulation measurements conducted on fish, resulted in significant values in vertebrates but invertebrates exhibit slightly higher values (Flint et al., 2012). For the last 20 years there have also been reported hundreds of studies about detectable concentration of BPA in biota (Geens et al., 2012). Specifically in algae, invertebrates and fish concentration of BPA fluctuated between 0.04 and 0.4 μg L^{-1}, while in humans 0.1 to 10 mM, respectively (Saiyood et al., 2010).

Environmental concentrations of BPA

BPA is detected mainly in water in wastewater treatment plants (in the effluent and sludge), the inflow of septic tanks in landfills and surface waters. Concentrations of this pollutant in water and sediment samples, depend on the emitting source and the time of sampling. A lot of data have been collected and the differences in concentrations are important. Bisphenol A concentrations' range varies from 0.09 to 11.8 g per liter in WWTP influent and 0.006 to 4.09 g per liter in the effluent according to Deblonde et al. (2011). In surface waters BPA concentration has been found greater than 20 μg L^{-1}, in river sediments was detected in quantities exceeding 1.63 mg kg^{-1} (Kabir et al., 2015) and finally in the landfill ranged between 0.07-269 mg L^{-1} (Joseph et al., 2011). Research reports on environmental concentrations of BPA are summarized in Table 3.

Table 3. Summarized BPA concentration levels reported in literature in influents.

Influent samples	Range of concentrations	References
Municipal wastewater	160–281 μg L^{-1}	(Toro-Velez et al., 2016)
Surface waters	0.0005–0.41 μg L^{-1}	(Fromme et al., 2002)
Sewage sludge / sediments	0.004–1.363 mg kg^{-1}	(Fromme et al., 2002)
River sediments	1.63 mg kg^{-1}	(Kabir et al., 2015)
Wastewaters	0.14 μg L^{-1} –28.1 μg L^{-1}	(Al-Rifai et al., 2007)
Wastewater Treatment Plants	0.088-11.8 μg L^{-1}	(Deblonde et al., 2011)

BPA degradation processes can be abiotic (through hydrolysis, oxidation and photolysis). Biodegradation of BPA can be achieved with the use of bacteria, which are present in wastewater treatment plants and rivers. The removal rate in the effluent wastewater treatment is estimated more than 90%, while the elimination half-lives observed in smaller rivers was five days. In surface waters with high BPA concentrations algae underlined demonstrated a BPA removal efficiency up to 63%. Another operational parameter for the removal of this pollutant is temperature. For temperature variation between 30 and 20 °C BPA half-live in river water was 4 and 7 days, respectively. At lower temperatures, BPA degradation rate was only 20% in a period of 20 days. Aerobic and anaerobic conditions also show great influence on the degradation of BPA. In aerobic conditions, rapid removal of BPA was observed at a rate greater than 90%, while under anaerobic conditions removal was less than 10% over 10 days (Kang et al., 2006).

2.1.3. Pharmaceuticals and Personal Care Products

Pharmaceuticals and personal care products (PPCPs) which contain diverse organic groups, such as antibiotics, analgesics, lipid regulators, psychotropic drugs, beta-adrenergic blockers, steroid hormones and personal care products such as cosmetics, perfumes, insect repellents and preservatives, are one of the largest and most significant categories of EOCs (Wang and Chu, 2016). Representative compounds of PPCPs are antibiotics, which are included in antimicrobial category of drugs and are the most widely used in modern human life. They are used to eliminate bacteria without causing damage to the host. Depending on their origin are classified as natural 10%, the 29% is classified as semisynthetic (derivatives) and the rest 61% as synthetic (Bade et al., 2010). Another classification is according to the chemical structure, their mechanisms of action, action spectrum and the route of administration. Among the classifications, the most frequently applied criterion is the mechanism of action. Acording to the latter, the most common groups are :b-lactams, sulfonamides, monobactams, carbapenems, aminoglycosides, glycopeptides, lincomycin, macrolides, polypeptides, polyenes, rifamycin, tetracyclines, chloramphenicol, quinolones, and fluoroquinolones (Gothwal and Shashidhar, 2015).

Antibiotics in the environment

The ecological effects of antibiotics have attracted increasing attention from both public and environmental fields due to its widespread usage (in medicine, veterinary/aquaculture for the prevention and treatment of bacterial infections and as development accelerators) and their improper disposal. These compounds are released mostly through urban wastewater and many of them can be further spreaded through the water cycle, even reaching drinking water, due to their hydrophilic character and low removal at wastewater treatment plants (WWTPs). This fact has initiated a huge scientific effort to better understand the occurrence and fate of the most commonly administered PPCP compounds in urban and hospital wastewaters and assess their potential environmental effects (Noguera-Oviedo and Aga, 2016). Repeated delivery of pharmaceutical compounds, mostly antibiotics and their metabolites in the environment, even at trace levels, are associated with a number of negative effects to the health of aquatic life and human beings, including short-term and long-term toxicity, development of pathogen resistance (Zuccato et al., 2006) and decrease in plankton diversity (Richards et al., 2004). Toxic impacts on human health, such as proliferation inhibition for the growth of human embryonic cells and degenerative and inflammatory reactions were observed. At present, information available concerning the ecotoxicology of pharmaceuticals is weak and the potential risks associated with the presence of pharmaceuticals in aquatic environments are still under debate (Richards et al., 2004; Santos et al., 2010). The most important issue of antibiotic release into the environment is related to the development of antibiotic resistance, which has resulted in the reduction of

therapeutic potential against human and animal pathogens. It is not the fact that the presence of antibiotic resistance was never seen before in the natural environment, but it was associated only with some bacterial strains, as resistance is an important process of evolutionary mechanisms. The release of antibiotics into the environment resulted in developing its resistance bacterial gene and other resistance genetic material (Antibiotic Resistance Genes, ARGs) (Noguera-Oviedo and Aga, 2016). The aforementioned antibiotic resistance genes increase in cases of untreated hospital and household waste and are also found in human pathogens and pristine environment, and now these genes can persist and spread even in the absence of antibiotics (Garcia-Rodríguez et al., 2014). Finally, even in low concentrations, antibiotics downgrade the quality of drinking water and therefore have adverse effects on human health leading to chronic diseases (Rivera-Utrilla et al., 2013). Among the antibiotics, ciprofloxacin (CIP) and sulfamethoxazole (SMX) are commonly detected in aquatic environment (Johansson et al., 2014; Lindberg et al., 2005).

2.1.4. Ciprofloxacin

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid) or CIP with molecular formula $C_{17}H_{18}FN_3O_3$ is part of the fluoroquinolone group (FQ) of antibiotics (Van Doorslaer et al., 2014). CIP targets the DNA gyrase of bacteria and so inhibits cell replication. Quinolones inhibit DNA synthesis by blocking type II topoisomerases (DNA gyrase and topoisomerase IV), the enzymes that control DNA supercoiling. DNA gyrase is crucial for inducing negative supercoiling of DNA, while topoisomerase IV functions as a decatenase (Rodríguez-Martínez et al., 2011). CIP's octanol-water distribution coefficient is equal to 0.28 (log K_{ow} = 0.28) in a solution with pH equal to 7.04, indicating high level of solubility in water (30 g L^{-1} at 20 °C) and low level in fats, respectively (Table 1). This value of log K_{ow} also reveals hydrophilic nature of CIP, non-binding to the solid phases of aquatic ecosystems (Kosma et al., 2014; Syranidou, 2016). CIP are applied in medical and veterinary field in order to confront bacterial infections as is active against a broad spectrum of Gram-negative and Gram-positive bacteria (Girardi et al., 2011). Moreover, CIP is considered the principle drug among therapy quinolones, particularly to combat gram-negative infections (Rodríguez-Martínez et al., 2011). According to the American Society of Health-System Pharmacists, the most commonly medical uses of CIP are for treating a wide variety of bacterial infections, including chest infections, urine infections, prostatitis, infections of the digestive system, bone and joint infections, and some sexually transmitted infections. Particularly, these antibiotics act against *Enterobacteriaceae*, such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp. with maximum efficacy against *Pseudomonas aeruginos* and with perspective to be a valuable candidate for therapy of neutropenic patients.

Sources and release paths in the environment

Despite CIP benefits in combating bacterial infections and their widely use in medical applications, a growing concern in scientific community has risen about potential adverse impacts in biota and human health as a result of their continuous release into the environment (Weber et al., 2011), especially though wastewater. CIP is one of the most frequently detectable chemical compound in Europe's, North's America, Asia's and Australia's wastewater treatment plants (Loos et al., 2013;Watkinson et al., 2007) and reports mention concentration values of 40-3353 ng L^{-1}, 110-1100 ng L^{-1}, 42-720 ng L^{-1} respectively (Gavrilescu et al., 2015). Larsson et al. evaluated concentrations up to 31 mg L^{-1} in the effluents of WWTP for pharmaceutical industries in India. Notably, detected concentration of CIP is generally higher in hospital effluent rather than in raw inflow of wastewater treatment plants (Varela et al., 2014). In general, even if CIP concentrations in the environment range from ng L^{-1} to mg L^{-1} (Girardi et al., 2011), their fate and effects on the ecosystem are yet unknown.

Furthermore, according to bibliography, studies had reported that the quantity of resistance genes is increasing rapidly at wastewater treatment effluents, resulting to a spread of these genes at surface water, a reduction in water quality and therefore an increased risk for fauna and humans (Rodriguez-Mozaz et al., 2015). The average concentration of CIP in Spanish and Chinese surface waters was reported to be 3 ng L^{-1} and 653 ngL^{-1}, respectively. Additionally, CIP was found to be the highest among all antibiotics with a mean concentration of 323.75 ng L^{-1} due to agricultural activities and/or infiltration of poorly treated water (Gothwal and Shashidhar, 2015). It is worth mentioning that CIP was also found in aquatic animals and birds in the range of 17.8-167 mg kg^{-1} (Li et al., 2012). According to the bibliography, CIP is frequently detected in the environment and is also proven to be genotoxic (Kümmerer, 2009). CIP, as all FQs, is excreted unmetabolized up to 70% and, when released in the environment, it can promote resistance formation on microbial populations thus causing adverse effects on aquatic life (Van Doorslaer et al., 2014). The presence of CIP in soil and fresh water sediments provokes the formation and the activity of microbial populations (Van Doorslaer et al., 2014). Genotoxic substances represent a health hazard to human organisms and it is proved that the genotoxicity of hospital wastewater effluents, could be attributed to the existence of CIP (Sturini et al., 2012). Accumulation of these compounds originates either through sewer system via human's secretion after antibiotics consumption or through leaching from sinks or toilets. Another provenance of FQs is from hospital waste due to its widespread clinical use (Van Doorslaer et al., 2014) and from expired and unused drugs disposed of in drains etc.

Additionally, when antibiotics are granted to fauna for medical reasons and then are defecated on the ground or directly into surface waters, this could enhance the risk of environmental pathogenesis not only in the soil but in water as well, because of CIP's hydrophilic character

(Rivera-Utrilla et al., 2013)(Rivera-Utrilla et al., 2013). In tap water samples of two Chinese towns, namely four FQs were detected by Gothwal et. al (2015). Among them traces of CIP ranged from 1.0 to 679.7 ng/L and 2.0 to 37 ng/L, respectively.

Mechanisms and findings in the environment

It is an emerging issue for environmental science and engineering to achieve effective removal of pharmaceuticals, along with other priority pollutants, from wastewaters before their discharge. Mechanisms including sorption, photo-degradation, and oxidation are recognized as the main elimination pathways for these compounds. Biodegradation of CIP is an innovative and difficult procedure rarely reported, with only a few bacteria to be able to biotransform CIP.

Knowledge in the field of microbial dissipation is inadequate according to a recent review study reported by Liao et al. (2016). The study pinpointed CIP as the sole carbon and nitrogen source for the microbiota and some possible degradation paths were described. Furthermore, during wastewater treatment, 80–90 % of CIP is removed via sorption to sludge through stabilization process (Girardi et al., 2011). The sorption of antibiotics in the soil is fast as it reaches equilibrium in few hours and is significantly governed by pH, organic matter, and mineral content in soil. It is weakly sorbed to minerals as compared to organic matter. Strong adsorption for CIP has been reported toward clay than to minerals like montmorillonite, illite, and kaolinite. Strong sorption to sludge also results in the releasing of CIP through biosolids to agricultural crops, posing a serious risk to animal health and people (Le-Minh et al., 2010). In soil reported concentrations ranged from 0.37 to 0.40 mg kg^{-1}. Despite the aforementioned possible CIP degradation mechanisms, environmental half-life time sorbed on soil matrices are, compared to sediment matrices, up to 200 times longer (Van Doorslaer et al., 2014). Oxidative degradation can occur by direct reaction with the applied oxidant or by producing highly reactive secondary chemical formulas. The process of oxidation involves either the use of a strong oxidizing agent (hydroxyl radicals, ozone, potassium permanganate, and chlorine) or processes which enhance the formation of free radicals (ozonation, Fenton oxidation, heterogeneous photocatalysis with TiO_2 and sonolysis) (Shinde et al., 2013). On the other hand, photodegradation of CIP is another technology with high efficiency and their half-lives are dependent on light intensity, pH, concentration level, phosphorus level, and the presence of organic matter.

Ozonation is also very effective in the removal of CIP. In this process, the ratio of molecular ozone over hydroxyl radicals, concentration of organic matter, and the corresponding reaction kinetics actually decides the oxidative pathways of antibiotics. More than 95% removal was observed in river water within 1.3 min at an ozone dosage of 7.1 mgL^{-1} (Gothwal and Shashidhar, 2015).

2.1.5. Sulfamethoxazole

Sulfamethoxazole (4-Amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide or SMX with molecular formula: $C_{10}H_{11}N_3O_3S$) is part of the sulfonamide group of bacteriostatic antibiotics. SMX targets the abeyance of hydrofolic acid and therefore folic acid creation, since this chemical compound is necessary for the survival of the bacteria, and contributes to their development and proliferation (Avisar et al., 2009). Thus, SMX is extensively used in medicine and veterinary in order to confront bacterial infections. Particularly, these antibiotics are excreted from the body without changes in a quota of 15% and active against *Streptococcus, Staphylococcus aureus, Escherichia coli, Haemophilus influenzae* and oral anaerobes. The most common use of SMX is to combat bacterial infections such as urinary tract infections, bronchitis, and prostatitis and is effective against both gram negative and positive bacteria such as *Listeria monocytogenes* and *E. coli* (Ednie et al., 1998). The octanol-water distribution coefficient of SMX is equal to 0.89 (log K_{ow} = 0.89) indicating low level of solubility in fats and high level in water (2800 mg L^{-1} at 20 ^{o}C), which increases at acid environment with values of pKa between 1.85-5.6. This value of log K_{ow} also reveals hydrophilic nature of SMX and its trend in non-binding to the solid phases of aquatic ecosystems (Kosma, Lambropoulou, & Albanis, 2014;Syranidou, 2016).

Sources and release paths in the environment

The rampant usage of drugs has made their occurrence ubiquitous in the environment and almost the whole of the world has acknowledged their presence in natural and artificial systems. Soil, sediment, sludge, groundwater, wastewater, tap water, surface water (lakes, streams, rivers and marine environment), plants and aquatic animals have been reported to be contaminated by antibiotics. Main resources of SMX are sewage disposal systems and WWTPs (Polesel et al., 2016).

Elimination of SMX was also investigated. Larcher and Yargeau (2012) have been reported up to 90% removal of SMX using conventional methods in wastewater treatment plants. Biodegradation of SMX is not affected by pH and temperature, resulting a low efficiency of removal techniques which are associated with these parameters such as hydrolysis. Research in pilot scale MBRs revealed a quota of 52-70% removal independently of the initial concentration; while 90% overall removal was achieved in a conventional WW treatment (Larcher and Yargeau, 2012). Additionally, the SMX easily degraded with the use of solar irradiation, in aqueous solutions with half-life time 2.4 days in winter and the rate of photodegradation was increased by the presence of humic acid (Andreozzi et al., 2003). Xekoukoulotakis et al., mentioned, also, that process performance of SMX mineralization is affected by several factors, namely irradiation time, photocatalyst type and loading, the presence of electron acceptors and the solution pH (Xekoukoulotakis et al., 2011).

Environmental concentrations of SMX

Several concentrations of SMX have been reported the last twenty years all over the world with higher values recorded in China, some exceeding 4870 ng L^{-1}. The concentration may vary but the percentage of detection in wastewater often reaches 100%. In Europe, North America, Asia and Australia, the recorded concentrations of SMX in effluence wastewater treatment were 91-794 ng L^{-1}, 5-2800 ng L^{-1} and 3.8-1400 ng L^{-1}, respectively. Furthermore, the detected concentrations of SMX in surface water were between 0.5 and 4 ng L^{-1} (Gavrilescu et al., 2015). High values of SMX have been also observed in groundwater in urban areas in Spain and China due to municipal landfilling (Jurado et al., 2012). Eighteen types of sulfonamides' concentrations ranging from 0.01 to 3460.57 ng L^{-1} were observed in groundwaters of Spain. Manure was the main source of SMX contamination, since sulfonamides are closely related to livestock veterinary practices. Concentration of SMX in seawater fluctuated between 0.51–6.30 ng L^{-1}, whereas in soil and sediments was 0.49 mg kg^{-1} (Gothwal and Shashidhar, 2015).

2.2.Phytoremediation of organic pollutants

2.2.1. Introduction

Environment is variously affected by plants either through substance intake or by producing compounds through the primary and secondary metabolism. This physicochemical interaction of plants with the environment can be used for the rehabilitation of areas, contaminated with organic compounds. Phytoremediation is an *in situ*, low-cost practice that has been applied for treatment of soils, sediments, wastewater or shallow aquifers, during the last 20 years (Reichenauer and Germida, 2008; Schwitzguébel, 2015). The term phytoremediation refers to multiple technologies that use photoautrophic vascular plants for the cleaning up of contaminated sites from organic and inorganic pollutants. The more general term "phytotechnology", is often used to include other methods aims to combine agricultural, forestry and horticulture experience, with functional integration of environmental sciences. For instance, constructed wetlands or new advances in -omics (metagenomics, metatranscriptomics, metaproteomics, metabolomics) (Reichenauer and Germida, 2008; Thijs et al., 2016).

2.2.2. Mechanisms of phytoremediation

Phytoremediation implements natural processes occurring in plants including absorption of water and compounds, metabolism into the plant, release of inorganic and organic compounds (such as mucigels, organic acids, dead cell material) in soil or substrate and physical and biological effects of the plant roots (Dordio and Carvalho, 2013; Thijs et al., 2016). There are 13 essential mineral nutrients (N, P, K, Ca, Mg, S, Fe, Cl, Zn, Mn, Cu, B and Mo) for plant growth that can directly be taken up by the plants (Hänsch and Mendel, 2009). Despite nutrients, plants are able to uptake other inorganic minerals (Pb, Cd, As, etc.) or organic pollutants. Based on the different processes involved, phytoremediation methods are classified as following (Etim, 2012; Vangronsveld et al., 2009):

- Phytodegradation (or phytotransformation): adsorption of contaminant by the plant and then cleavage / degradation of the tissues.
- Rhizodegradation or enhanced rhizosphere biodegradation: decomposition of organic contaminants in soil through microbial populations growing in the root zone.
- Phytovolatilization: metal and organic compounds adsorption by plants, conversion to volatile forms and release through leaves via evapotranspiration processes.
- Phytoextraction or Phytoaccumulation: uptake and translocation of metals in plants.

- Phytostabilization: immobilization to the soil, or limitation of the migration of contaminants to the groundwater.
- Rhizofiltration: adsorption or precipitation of inorganic contaminants from water onto root system.

2.2.3. Phytoremediation of organic pollutants

Absorption of organic compounds by the plants could be affected by several parameters, such as molecular weight of the compound, octanol-water partition coefficient, hydrophobicity, concentration and acidity constant (pKa) (Newman and Reynolds, 2004; Stottmeister et al., 2003). Generally speaking, compounds with $\log K_{ow}$ values between 1 and 3.5 can be more easily absorbed by plant roots. It is believed that their degradation rate is not high enough to prevent them from entering into the xylem. After they enter into the plant they may be degraded to metabolites that in some cases could be toxic (Vangronsveld et al., 2009).

Hydrophobic components with $\log K_{ow}$ values greater than 3.5 either have low water solubility or bind as strongly to the surface of roots, hence they cannot be taken up by the plant easily. Though, recent publications indicated that some plants are able to uptake specific hydrophobic xenobiotics with $\log K_{ow} > 4$. For instance, the uptake of PCBs with $\log K_{ow}$ 4.5-8.2, by species of the *Cucurbitaceae* family (Schwitzguébel, 2015). On the other hand, hydrophilic substances with $\log K_{ow}$ values lower than 1 are highly polar and very soluble in water are not sufficiently sorbed onto roots or translocated within the plant (Reichenauer and Germida, 2008). Among physical and chemical characteristics of soil and the pollutant, plant type is addressed as another factor influencing phytoremediation.

Organic pollutants can be effectively degraded by microorganisms through the process of rhizodegradation and phytodegradation. Organic compounds may be transported across cell membranes. Volatile ones can be absorbed from the roots and released through the leaves by transpiration. Some of the non-volatile compounds are degraded or transformed to non-toxic, via enzymatic activity (Afzal et al., 2014).

An important issue in phytoremediation, is also the high concentrations of pollutants which tend to inhibit plant and root growth due to oxidative stress, reducing in this way the efficiency of the technology (Gerhardt et al., 2009). However, other studies have shown that removal efficiency is not always negatively affected with the increase of pollutant's concentration. Moreover, polluted soils tend to be poor in nutrients and have a reduced microbial diversity, which in turn contributes to the lower biomass growth, and reduce remediation efficacy (Fink et al., 2009)

2.2.4. Phytodegradation and Rhizodegradation

Figure 2 illustrates the mechanism involved to the removal of organic contaminats by plants Among the phytoremediation mechanisms, phytodegradation and rhizodegradation are the most prevalent in dealing with organic pollutants. Plants are naturally equipped with a complex and versatile enzymatic system to diminish phytotoxicity. In phytodegradation, the pollutant is degraded by plant enzymes, while in case of rhizodegradation, enzymatic degradation is taken place within the plant root zone, or rhizosphere (Etim, 2012; Gerhardt et al., 2009). Uptake of pollutants by plant roots is different from organics and inorganics. Organics uptake is driven by simple diffusion, because they are not equipped with membrane transporters such as in the case of natural compounds.

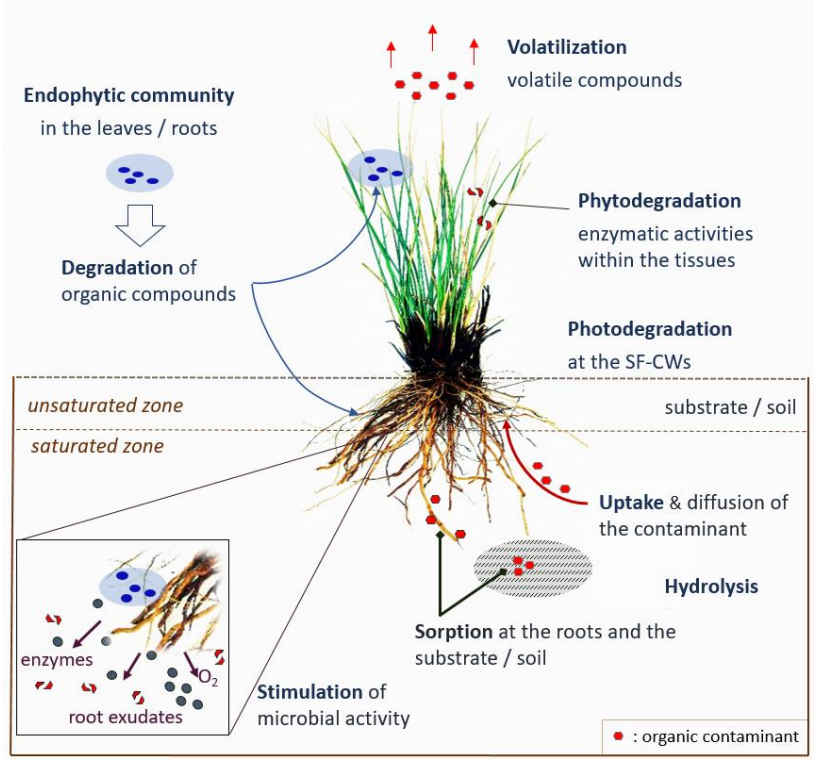


Figure 2. Schematic representation of the contribution of *Juncus* spp. on organic contaminants removal from soil, groundwater or wastewater. Prevailing remediation mechanisms and schematic representation of the effect of plant associated microorganisms and root secretions (Syranidou et al., 2016).

According to Sandermann (1992), metabolization of organic compounds is occurred in three sequential steps: transformation, conjugation and compartmentation. In these phases, different enzymes are involved, namely: glutathione transferase, cytochrome P450, carboxylesterase, O- and N-glucosyl transferase and O- and N-malonyl transferase. Accordingly, detoxification of organic pollutants is associated with exporting into the cell vacuole or the extracellular space and integration into the cell membrane. However, these paths do not play a fundamental role in

the degradation, in comparison with microorganisms (Reichenauer and Germida, 2008; Stottmeister et al., 2003).

Rhizodegradation, also known as phyto-stimulation, is the degradation of contaminants in the rhizosphere (area of soil surrounding the roots of the plants) by means of microbial activity, which is stimulated by the presence of plant roots. Is one of the most effective process in which plants influence the degradation of organic pollutants, especially in the presence of large and resistant compounds. In this case, complex interactions associated with the roots, their secretions, the soil in the rhizosphere and microorganisms, contribute to the degradation of organic substances to non-toxic or less toxic compounds.

Rhizodegradation occurs naturally through the release of various compounds released from roots (such as flavonoids and coumarins), which stimulate the growth and activity of xenobiotic degradation bacteria. Moreover, development of root system promote aeration of rhizosphere, which potential enhance degradation of the organic pollutants. Interestingly, rhizosphere microorganisms was found to be exceptionally increased by the presence of specific plant species (Gerhardt et al., 2009). As stated, rhizosphere soil has 10-100 times higher microbial biomass, than the bulk (non-rhizosphere) soil (Lynch, 1990).

A special rhizodegradation and more widely, phytoremediation aspect, is the application of constructed wetland systems that take advantage of the above processes in order to treat wastewater, contaminated with organic xenobiotics.

2.3. Constructed Wetlands

2.3.1. Introduction

The occurrence of organic micropollutants in the environment has gained growing concerns, as a result of the possible undesirable effects it may causes. Unfortunately, conventional wastewater treatment plants only partially remove xenobiotics from wastewater, since they are not currently designed to cope with this type of contamination (Avila et al., 2013). Therefore, a number of technologies have been addressed and different processes have been tested in order to decrease the organic pollutant discharge into the environment (Tore et al., 2012).

Constructed wetlands (CWs or treatment wetlands) are presented as a potential alternative for wastewater treatment, contaminated with organic xenobiotics. It is a low-cost, eco-technological biological wastewater treatment technology that is designed to mimic processes found in natural wetland ecosystems (Kalogerakis and Christofilopoulos, 2015). They consist of a shallow basin, filled with some sort of filter material (substrate), usually sand or gravel and

planted with vegetation tolerant to saturated conditions (emergent, submerged or floating) (Liu et al., 2016). In these treatment systems, wastewater is introduced into the basin, then is percolated by gravity inducement over the surface or through the substrate, and treated wastewater is discharged (UN-HABITAT, 2008).

Therefore, purification of wastewater could be achieved by a wide variety of physical, chemical and (micro) biological processes: sedimentation, filtration, precipitation, sorption, plant uptake, microbial decomposition and nitrogen transformations among plants, substrate or soil (Rousseau et al., 2004). Soil acts as the main supporting material for plant growth and biofilms. Moreover, the soil matrix has a decisive influence on the hydraulic processes. Both chemical soil composition and physical parameters such as grain-size distributions, interstitial pore spaces, effective grain sizes, degrees of irregularity and the coefficient of permeability are proven to be crucial factors, influencing the biological treatment systems. (Ahmed et al., 2016). Some commonly used helophytes are common reed (*Phragmites australis*), cattail (*Typha* spp.) and bulrush (*Scirpus* spp.), which all are characterized as water-tolerant macrophytes and even are rooted in the soil, they emerge above the water surface (Rousseau et al., 2004).

Constructed wetlands are classified as surface flow (SF) and subsurface flow (SSF). There are two principal types within SSF-CWs: horizontal flow (HF or HSSF) and vertical flow (VF or VFSS). Moreover combination of different CWs produces hybrid systems, taking advantage of the characteristics of each different system (Ahmed et al., 2016).

2.3.2. Horizontal Flow CWs

The use of CWs for urban wastewater treatment has gained growing interest over the last decades. Horizontal subsurface flow constructed wetlands (HSF-CWs) are a widely applied technology. A typical HF CWs as it is observed in Fig. 3 consist of a bed, usually gravel, which is sealed by an impermeable layer and is planted with wetland vegetation. The wastewater is applied at the inlet and flows horizontally through the porous medium to the outlet zone. Biotic and abiotic processes are carried out during this route.

Removal of organic matter in HSF-CWs is attained by aerobic and anaerobic processes (such as denitrification), filtration and sedimentation. Anoxic conditions are prevailed in the filtration bed, because of the continuous saturation. Aerobic processes are occurred in a thin layer close to the surface due to oxygen diffusion from the atmosphere, and to specific zones in the rhizosphere, as a result of oxygen release by the roots. In cases, the inflowing wastewater is also of supplying the wetland system with oxygen (Vymazal, 2004).

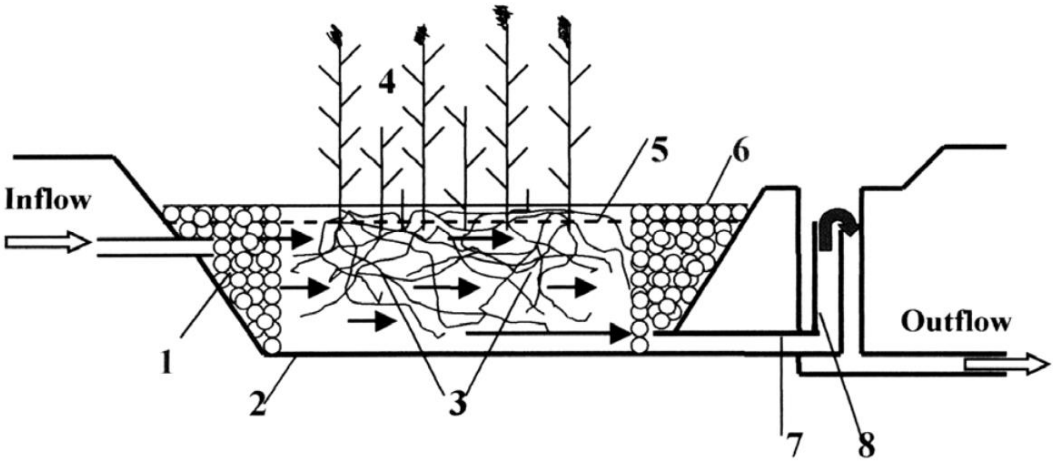


Figure 3. A typical horizontal subsurface flow constructed wetland: 1) wastewater influent; 2) impermeable layer; 3) filtration bed; 4) vegetation; 5) water level; 6) outflow; 7) drainage pipe; 8) outflow, providing adjustment of water level (Vymazal, 2010).

In addition to biotic, several abiotic processes are involved in organics removal in CWs. Sorption to plant roots, substrate or biofilm in the rhizosphere, is considered of major importance. What is more, photodegradation, hydrolysis, redox reactions and volatilization, are reported to play a secondary role in organic contaminants removal (Dordio and Carvalho, 2013).

2.4. The role of vegetation

A fundamental role of plants in HSF-CWs is that provide substrate (root and rhizomes) for the growth of microbes, radial oxygen loss and nutrient uptake (Vymazal, 2010). Furthermore, they may contribute directly to chemical metabolism and reduce resuspension of sediments in wetlands. Moreover, plants are a key design factor since they stabilize bed surface, act as an anchoring surface for biofilm and release exudates and oxygen though their roots (Hijosa-Valsero et al., 2010b).

They contribute to the attenuation of many organic pollutants such as EDCs and PPCPs from wastewater, not only through direct uptake but also by providing favourable conditions for rhizosphere microorganisms to grow (Garcia-Rodríguez et al., 2014). After being taken up into plant tissues, compounds might be degraded via the metabolism processes including a series of biochemical reactions. Stimulation of microbial populations, which is supported by the rhizodeposition products (such as mucigels, exudates, dead cell material), results in various biological processes to occur in the rhizosphere, as previously discussed. Studies have also demonstrated that plants provide an increased surface area not only for microbial activity, but for sorption as well (Stottmeister et al., 2003).

Most plant species in constructed wetlands are able to release oxygen around their root tips and on young laterals promoting the oxidative chemical processes of contaminants in wastewater and favor the development of aerobic microorganisms in the rhizosphere inducing more efficient biodegradation processes. The continuous release of oxygen from the root zones of plants might counterbalance the chemical and biological oxygen consumption in the rhizosphere (Li et al., 2014b)

2.4.1. Plant selection criteria

Plant selection for application in CWs could play a strategic role in system's performance, since vegetation is considered an essential component of design consideration. However, only a few species have been widely used in constructed wetlands and the practical planning and maintenance of plantations is still premature, as we lack the appropriate knowledge to direct these endeavors (Shelef et al., 2013). Nonetheless, the positive effect of vegetation on CW performance has been often revealed. For instance, Vymazal (2011) summarizing multiple articles comparing treatment efficiency of vegetated versus non-vegetated CWs, deduced that 90% of the reports performed a positive effect of at least some plants on some water quality parameters(Shelef et al., 2013). Macrophytic species are considered to be appropriate for CW applications, since they have fast growth rate, rapid establishment usually by clonal propagation, high biomass production and good tolerance to clogging conditions (Brisson and Chazarenc, 2009).

2.5. Helophytes - *Juncus acutus* L.

Many species was found to be able to grow in contaminated sites and cope with significantly high concentration of pollutants. Though they were inappropriate for field applications due to their low biomass production and the poor growth rate (Vangronsveld et al., 2009). Helophytes have been presented as appropriate plants for phytoremediation and they have been widely utilized in CWs (Wiessner and Kuschk, 2006). In general, their main contribution in CWs is that they alter the microhabitat, thus making it suitable for contaminant removal through several mechanisms, acting as nutrient pumps and as filters to suspended particles (Syranidou et al., 2011; Vymazal and Březinová, 2016). According to the literature, they work best in semi-natural wastewater treatment systems, due to physiological characteristics as afore-mentioned, that render them capable of surviving under the extreme conditions they are exposed (Stottmeister et al., 2003).

The genus *Juncus* comprises of helophytes, perennial or rarely annual, marsh species with a wide distribution (Vymazal, 2013). They tolerate water-clogged environments while the majority of the species are characterized as halotolerant helophytes and few species as obligatory halophytes. In accordance with the other wetland plants, they release high amounts of oxygen in the rhizosphere but in a species-specific pattern (Blossfeld et al., 2011). For example, *J. inflexus* and *J. effusus* create a constant cone-shaped oxic zone around their root tips and a diurnally changing oxic zone around their lateral roots. On the other hand, *J. articulatus* release constantly oxygen along the roots. (Böhling et al., 2002). Many authors suggested that some *Juncus* species are appropriate plants for remediating areas polluted with Hg and Zn, while other species are not so efficient in treating organics such as PAHs and benzo(a)pyrene (BaP).

During the past decades, numerous similar phytoremediation studies with halophytes have been conducted, some with significant results. Although, application in real conditions was not always encouraging. As a general agreement, research on the appropriate macrophytes for specific CW applications, as long as evaluation of their contribution to wastewater treatment is still needed. The challenge is not just the interpretation of the mechanisms and data obtained, but also the implementation of an efficient system, capable of dealing with a realistic environmental scenario.

CHAPTER 3. EXPERIMENTAL DESIGN

3.1. Small- scale experiments

In this chapter pot-scale and hydroponic experiments testing the efficiency of halophytes to treat contaminated water and soil, are presented. Artificially spiked tap-water was used in all experiments that are distinguished in three phases: i) pot experiments with soil substrate in growth chambers, ii) pot experiments with soil substrate in environmental, non-controlled conditions and iii) a hydroponic, glass house experiment. Apart from the hydroponic experiment that a complex mixture of contaminants (organic and inorganic) was added, in the first two, BPA was the sole spiked compound.

Plant materials and culture conditions

J. acutus plants were collected from the Souda bay of Chania, whereas *T. parviflora* plants were purchased locally (Galanis, Chania, GR). Both species were grown in plastic pots until the beginning of each experiment. Weighing of plants and measurement of root and shoot length, assured the similarity of the replicates. Experimental set up included the use of ceramic pots, polyethylene bags (PEB) and glass vessels, according to the needs of each case. Four different small-scale experiments were conducted. The first three were in soil substrate with BPA as the only spiked pollutant. The forth was conducted in hydroponic conditions. In the latter that aimed to withdraw soil effect from the remediation procedure, the effect of mixed contamination with the presence of antibiotics and heavy metals, was also studied.

3.1.1. Small- scale experiments in soil substrate

PE bags in growth chambers

In this experiment, soil mass used was approximately the 1/3 of the following experiments mass. BPA mass in both aqueous and soil phase were determined and total removal of the compound after 6 days of treatment was estimated. Small pot experiments were performed in plant growth chambers in order to evaluate the contribution of plants on the removal of BPA, taking into consideration the rhizosphere effect. PE bags experiment with eight replicates per treatment, as follows:

(1) Non-planted, spiked with BPA;

(2) Planted, spiked with BPA.

Plastic pots (5 cm x 5 cm x 8 cm) were lined with polyethylene bags to avoid cross-contamination and loss of water and each pot was filled with 285 g of soil (dry weight). Plants

were weighted, planted and watered to 120% of water holding capacity by adding 100 mL of tap water and maintained at this moisture. Each pot was spiked with 3.45 mg BPA at day 0 (total duration of the run was 6 days) and kept in a growth chamber at 26 °C temperature and 16 h day / 8 h night time period program. Three out of eight plants of each treatment were sacrificed for each sampling (at day 1 and day 6). At first they were weighted to determine evapotranspiration, then unplanted and soil was separated carefully. At day two, 15 and 35 ml of tap water were added in each replicate of the non-planted and planted treatments, respectively. Aqueous phase samples were centrifuged for 2 min at 3500 rpm and BPA concentration in the supernatant was determined by HPLC, after filtering through a membrane filter of pore diameter 0.45 μm.

Figure 4. *J. acutus* plants before planting (left) and during PEB experiment in the growth chamber (right).

J. acutus in pots system in growth chambers

The next phase of these experiments was designed to run in plant growth chambers, in order to allow constant environmental conditions. Species of genus *Juncus* was exclusively selected for this experiment, as it showed highest removal of BPA concentration, in comparison with *T. parviflora*. Moreover, as a hydrophytic species, *J. acutus* is better adapted to the water – saturated conditions of the experiment. The same type of ceramic pots with the drainage valve at the bottom, were set inside the chambers at 26 °C temperature and 16 h day / 8 h night time period program. Here, for practical reasons, soil and gravel masses were slightly changed at 1100 g and 250 g respectively. Each pot was spiked with 30 mg BPA at day 0 (total duration of the run was 6 days). Watering and sampling techniques were remained the same as previously. Tap water without spiked BPA, was added in each pot until water reach the soil surface. Water volume added was recorded and the infiltrate was collected after three hours and

water volume was recorded using a volumetric column. BPA extraction from soil was conducted in soil samples, collected from each pot at day 1 and day 6.

Figure 5: Overview of pot experiment with planted and non-planted treatments in the growth chamber

One plant was grown in each pot and there were three replicates for each treatment. The treatments were as follows:

(1) Non-planted, with BPA addition;

(2) *J. acutus*, no BPA addition;

(3) *J. acutus*, with BPA addition.

Simultaneously, bioaugmentation with *Sphingomonas* sp. strain TTNP3 was tested in these first two experiments, however results are not presented are not presented in this thesis.

T. parviflora and *J. acutus* in pot system under non-controlled conditions

In this experiment, contribution of the halophyte *Tamarix parviflora* was additionally investigated, in comparison with *J. acutus* and control treatments. It was conducted in non-controlled conditions, following almost the same experimental routine as the previous study. Total amount of BPA in the system, i.e. the amount absorbed by the soil and the amount dissolved in the aqueous phase, was estimated through the measurement of the aqueous phase and estimation of K_{SD}. Precisely, the later was achieved using mass balance equation (Equation 1), where $m_{BPA}^{Total}(t)$ the initial BPA mass added at each pot at time zero and C_L the measured concentration of the compound after remaining three hours in the pot system. Estimated K_{SD} for each replicate was then used to determine C_S, hence total BPA mass remaining in each pot.

Ceramic pots (13 cm in diameter, 11 cm in height, 1400 mL total volume) were purchased locally and a sampling port with a valve was introduced at the bottom. Each pot was filled with 400 g of gravel at the bottom and 1170 g of dry soil above. Soil used for the experiment was a mixture of soil collected from the field in Akrotiri, Chania and beach sand (prewashed), in order to increase the permeability and have rapid infiltration of the water. Maximum water holding capacity of 1200 g soil was measured at 0.29 L. Analysis of the soil mixture indicated a sandy soil, with pH value of 8.7, low organic matter (1.2%), total $CaCO_3$ 18.1% and electrical conductivity 0.53 mS cm^{-1}. Before the addition into the pots, soil was air-dried and sieved through a 3 mm sieve. The system was setup in the Technical University of Crete (35º32'00.40'' N 24º04'12.50'' E), as depicted in Fig. 6.

One plant was grown in each pot and there were three replicates for each treatment. The effect of bioaugmentation with *Sphingomonas* sp. strain TTNP3, was also investigated, notwithstanding it is not described in this thesis, since it is a part of another study from Syranidou (2016). The treatments, with three replicates each, were as follows:

(1) Control, without plant, with BPA addition;

(2) Control with *T. parviflora*, no BPA addition;

(3) Control with *J. acutus*, no BPA addition;

(4) *T. parviflora* with BPA; and

(5) *J. acutus*, with BPA.

Figure 6. Overview of the pot experiment (left) and details of the pot /sampling port design (right).

Pots were spiked with 150 or 300 μg BPA each (Table 4), diluted in 250 mL of tap water, at the first day of each run (total duration of the run was 3 days). In order to achieve better homogenization, contaminated water was recirculated twice, remained for two hours in the pot system and drainage was collected. The following two days, 150 mL of tap water without BPA, were added in each pot and the drainage was collected after two hours.

Table 4. Treatments tested at the two runs of pot experiment under non-controlled conditions and initial BPA doses applied at each replicate.

Treatment	1	2	3
Plant type	**No plants**	***T. parviflora***	***J. acutus***
BPA applied 1st run	150 μg (0.6 mg L^{-1})	300 μg	150 μg
BPA applied 2nd run	300 μg (1.2 mg L^{-1})	300 μg	300 μg

Calculations

BPA concentrations in the aqueous phase was measured by HPLC as described in previous section. In order to estimate the total amount of BPA in the system, i.e. the amount absorbed by the soil and the amount dissolved in the aqueous phase, one needs only to measure the concentration of BPA in the liquid phase (C_L). The concentration of the BPA absorbed in the soil (C_S) can be readily estimated, when no extraction of the compound from the soil was performed, using C_L and the partition coefficient K_{SD}, since distribution of BPA between the two phases is strictly dictated by the K_{SD} factor (Petoussi et al., 2014).i.e.

$$\boldsymbol{C_S = C_L K_{SD}} \qquad \text{(Equation 1)}$$

where C_L is in mg L^{-1} and C_S in mg kg^{-1}.

BPA mass in the soil can be estimated by applying the following equation:

$$C_S = \frac{(C_0 - C_L)\, V_L}{m_{soil}} \qquad \text{(Equation 2)}$$

where C_0 in mg L^{-1}, is the initial BPA mass in the aqueous phase, V_L in L, the volume of the liquid phase in the pot system and m_{soil} the soil mass in kg.

When BPA extraction from soil was performed, K_{SD} was obtained from equation (1), by substituting C_L and C_S with the mean measured values of all replicates. The mass of BPA remaining in the soil was estimated over time, using the following equation as a function of the

BPA concentration in the aqueous phase (as measured by HPLC), and the three known constants K_{SD}, m_s and V_L.

$$m_{BPA}^{Total}(t) = m_S(t) + m_L(t)$$

$$m_{BPA}^{Total}(t) = C_S(t)\, m_{soil}(t) + C_L(t)\, V_L$$

If we assume that thermodynamic equilibrium is attained quickly, by using equation (1), the total BPA mass remaining in the systems takes the following form:

$$m_{BPA}^{Total}(t) = C_L(t)\,(V_L + K_{SD}\, m_{soil}) \qquad \text{(Equation 4)}$$

where C_L (mg L^{-1}) is the concentration of BPA measured in the liquid phase at the last day of the experiment. BPA degradation rates were estimated by Least Squares calculations, taking into account soil mass in each pot (m_{soil} in kg). In general, the degradation rate of BPA is given by equation (5), as BPA is degraded in the liquid phase only:

$$\frac{d[m_{BPA}^{Total}]}{dt} = -k\, C_L\, V_L \qquad \text{(Equation 5)}$$

where k is first order degradation rate constant in d^{-1}.

If we assume rapid thermodynamic equilibrium, the above equation becomes:

$$\frac{d[C_L(V_L + K_{SD}\, m_{soil})]}{dt} = -k\, C_L\, V_L$$

$$(V_L + K_{SD}\, m_{soil})\frac{d[C_L]}{dt} = -k\, C_L\, V_L$$

$$\frac{dC_L}{dt} = -\left(\frac{k}{1 + K_{sd}\frac{m_{soil}}{V_L}}\right) * C_L$$

i.e.

$$\frac{dC_L}{dt} = -k' * C_L$$

Where k' is the apparent degradation rate constant of the compound.

$$k' = \frac{k}{1 + K_{sd}\frac{m_{soil}}{V_L}} \qquad \text{(Equation 6)}$$

Thus, in the case where thermodynamic equilibrium is attained, apparent BPA degradation kinetic constant k' in the aqueous phase, can be obtained from the slope of the linear plot of $ln\left(\frac{C_L}{C_L^0}\right)$ versus time, thus:

$$ln\left(\frac{C_L}{C_L^0}\right) = -k't \qquad \text{(Equation 7)}$$

In the hypothesis that thermodynamic equilibrium could not be rapidly reached, then C_S and C_L are not in equilibrium, and hence, in order to follow the total BPA degradation, we consider the following:

From the starting equation (5):

$$\frac{\mathrm{d}[\mathrm{m}_{\mathrm{BPA}}^{\mathrm{Total}}]}{dt} = -k\,C_L\,V_L$$

and given equation (3), we obtain:

$$m_{soil}\frac{dC_S}{dt} + V_L\frac{dC_L}{dt} = -k\,C_L\,V_L$$

$$V_L\frac{dC_L}{dt} = -k\,C_L\,V_L - m_{soil}\frac{dC_S}{dt}$$

$$\frac{m_{soil}}{V_L}\frac{dC_S}{dt} + \frac{dC_L}{dt} = -kC_L$$

$$\frac{m_{soil}}{V_L}dC_S + dC_L = -kC_L dt$$

$$\frac{m_{soil}}{V_L}\frac{dC_S}{C_L} + \frac{dC_L}{C_L} = -k\,dt$$

$$\frac{m_{soil}}{V_L}\left[\frac{C_S}{C_S}\right]\frac{dC_S}{C_L} + \frac{dC_L}{C_L} = -k\,dt$$

$$\frac{m_{soil}}{V_L}\left[\frac{C_S}{C_L}\right]\frac{dC_S}{C_S} + \frac{dC_L}{C_L} = -k\,dt$$

Integrating both sides we obtain:

$$\frac{m_{soil}}{V_L}\int\left[\frac{C_S}{C_L}\right]\frac{dC_S}{C_S} + \int\frac{dC_L}{C_L} = -k\int dt$$

Using the mean value theorem, the above equation becomes:

$$\overline{\left[\frac{C_S}{C_L}\right]}\frac{m_{soil}}{V_L}\int\frac{dC_S}{C_S}+\int\frac{dC_L}{C_L}=-k\,t \qquad \text{(Equation 8)}$$

where $\overline{\left[\frac{C_S}{C_L}\right]}$ is the average of $\left[\frac{C_S}{C_L}\right]$, which should be relatively constant and somewhat lower than K_{SD}.

Integration of Equation (8) yields:

$$ln\left(\frac{C_L}{C_L^0}\right)+\frac{m_{soil}}{V_L}\beta\;ln\left(\frac{C_S}{C_S^0}\right)=-k\,t \qquad \text{(Equation 9)}$$

Where, $$\beta\;=\overline{\left(\frac{C_S}{C_L}\right)}$$

Hence, by plotting $ln(C_L)+\frac{m_{soil}}{V_L}\beta\;ln(C_S)$ versus time t, the slope yields the unknown kinetic rate constant, k.

Finally, percentage removal of the organic contaminants was obtained using the following equation:

$$Concentration\;removal\;(\%)=\frac{C_{t1}-(1-\Delta V)\,C_{t2}}{C_{t1}}\;x\;100 \qquad \text{(Equation 10)}$$

Where C_{t1}, C_{t2} are the measured concentrations of the compound at the times t_1 and t_2, respectively ($t_1 < t_2$), σε mg L^{-1}.

3.1.2. Hydroponic experiment

Experimental setup

Juncus acutus plants were collected from the Souda bay (Chania, Crete, Greece), divided into plantlets of the same size (25 g ± 5), washed with fresh water and weighed, before placed into the vessels. Clean glass vessels (1.5 L total volume) were covered with aluminium foil and filled with 950 g of gravel. Plants were irrigated initially with 350 mL of 10% Hoagland's solution and then once a week water was added to each vessel to maintain a constant and equal for all replicates volume of 350 mL. The acclimatization period was 2 weeks. Ten treatments in total were performed in seven replicates to reduce uncertainty and presented in Table 5.

Table 5. Experimental design and concentration of spiked contaminants for each treatment at day zero

No.	Type	Treatment	Description	Nominal Concentration			
				CIP ($\mu g\ L^{-1}$)	SMX ($\mu g\ L^{-1}$)	BPA ($\mu g\ L^{-1}$)	Cr / Ni / Zn / Cd ($mg\ L^{-1}$)
1	Controls	A	no contaminant		-	-	-
2		CA	no plants	100	500	-	-
3		CH	no plants	100	500	500	20 / 40 / 250 / 1.5
4	Antibiotics	B	Low Concentration	1	5	-	-
5		C	Medium Conc.	100	500	-	-
6		D	High Conc.	10000	50000	-	-
7	Antibiotics + BPA + Heavy metals	E	Low Conc.	1	5	1	0.2 / 0.4 / 4 / 0.02
8		F	Medium Conc.	100	500	500	20 / 40 / 250 / 1.5
9		G	High Conc.	1000	5000	50000	250 / 300 / 1250 / 10
10	Heavy metals	H	High Conc.	-	-	-	1650 / 750 / 1250 / 15

Figure 7. Overview of the experimental setup of the hydroponic experiment inside the glasshouse. The seven replicates of each treatment are well distinguished in the picture

Meteorological parameters were monitored every 2 h, by a DT-171 Temperature and Humidity data logger. Experimental period was 28 days (November to December), where daylight was 9-10 h. In addition, pH was measured weekly for each replicate in order to access potential precipitation of the heavy metals stated. At the end of the experiment, the plants were harvested; leaves were separated from roots and washed once with tap water and twice with deionized water. Samples were grounded with a blender, followed by mortar in the presence of liquid nitrogen in order to attain a fine powder. Approximately two grams of plant roots and leaves fresh powder were weighed for organic compounds analysis within the plant tissues. The rest of plant biomass was oven dried at 70 ^{o}C for 48 h prior to being weighed for heavy metal analysis.

With respect to sampling procedure, every 7 days, 4 mL of the aqueous phase of each replicate was collected. Vessels containing plants, gravel and water were weighed to estimate water consumption and tap water was added in order to maintain equal water volume for each sample. Samples were filtered through a 0.45 mm syringe filter, in order to obtain a clear solution appropriate for injection to the HPLC.

Metal transfer within the plant

Translocation and distribution of metals in plant tissues was investigated by calculating several parameters. Removal by the plant (%) of the initial pollutant was estimated as the ratio of metal mass in the whole plant to the total mass of the metal, initially added in the nutrient solution. Namely:

$$\text{Removal by the plant (\%)} = \frac{C_L m_L + C_R m_R}{C_{sol} V_{sol}} 100 \qquad \text{(Equation 11)}$$

where: C_L and C_R are the concentrations of the metal accumulated in the leaves and roots (μg g^{-1}), respectively. m_L and m_R are the mass dry weight of the leaves and roots per plant,; and C_{sol} is the initial concentration of metal in the nutrient solution of volume V_{sol}.

Additionally, the bioaccumulation coefficient (BC), defined as the ratio of metal concentration in the whole plant (C_p) to the initial concentration of the metal in the nutrient solution (C_{sol}), (Kachout et al., 2009;Singh et al., 2010) was used in order to evaluate the portion of each metal that was transported from the aqueous phase to the plant. Namely,

$$BC = \frac{C_P}{C_{sol}} = \frac{C_L m_L + C_R m_R}{C_{sol}(m_L + m_R)} \qquad \text{(Equation 12)}$$

Furthermore, the ability of plants to translocate metals from roots to leaves was evaluated by the translocation index (TI), as the ratio of the total metal mass accumulated in the leaves, to the total metal mass accumulated in the whole plant (Vittori Antisari et al., 2015), namely:

$$TI\ (\%) = \frac{C_L m_L}{C_L m_L + C_R m_R}\ 100 \qquad \text{(Equation 13)}$$

3.2. Shallow Aquifer Rhizodegradation pilot

3.2.1. Description of the system

Two *J. acutus* plants, were collected from Souda Bay (Chania, Greece), were transplanted to the pilot and left for a month before the beginning of the experiments, to allow acclimation to the new environmental conditions. The system was setup in the Technical University of Crete (35°32'00.40'' N 24°04'12.50'' E) as depicted in Fig. 8 and simulates a shallow aquifer treating contaminated groundwater. In this regard, a water system was adjusted allowing a continuous contaminated groundwater inlet at the bottom of the pilot. A polyethylene tank of 1 m^3 was used as the primal section of the construction, which can be separated into three layers: the first one of 10 cm high at the bottom of the pilot consist of small size gravel and take hold of 0.116 m^3. Above there was a medium gravel layer of 0.058 m^3. These two layers work for the proper distribution of the influent to the main compartment of the unit, the third layer, consisting of soil equals with 0.98 m^3. The soil mass in the system was estimated 1,040 kg and the water volume 315 L, at total saturation. An external reservoir of 75 L capacity (working volume 60 L) was used for recirculating wastewater and as a contaminant's distribution tank. The pump was working continuously 24 h d^{-1} with a specific flow rate, according to the desired HRT of each experimental run, sending water from the external reservoir to the lower zone. Contaminated water was flowing from the bottom to the surface of the tank, (sampling point) passing through the rhizosphere. In the soil surface, treated water was overflowing back to the reservoir, after filtration in a small gravel section.

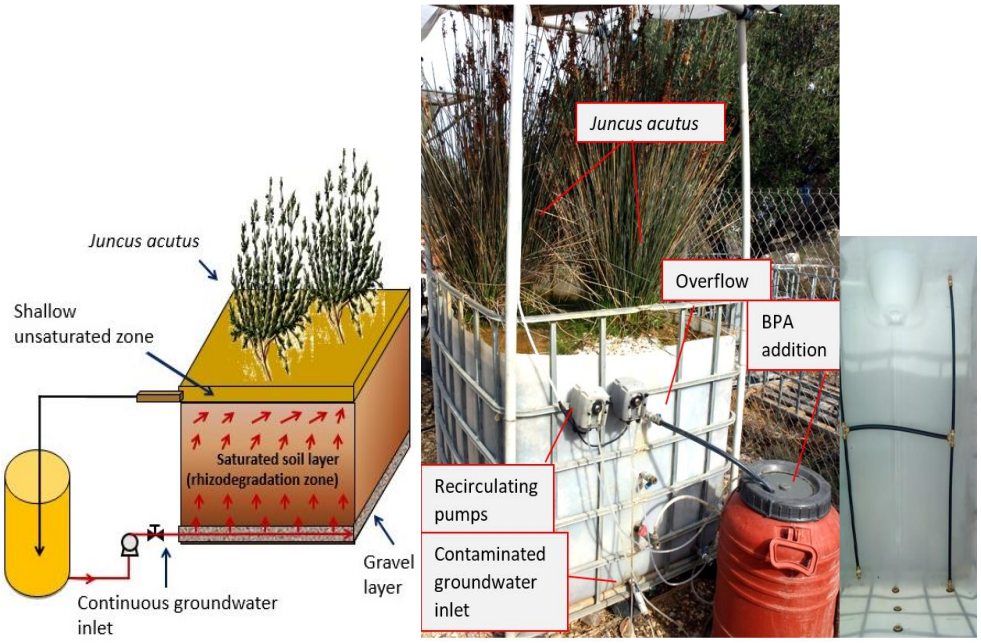


Figure 8. Layout of the pilot unit, showing the contaminated water flow. B: Picture of the rhizodegradation pilot and the related equipment C: Bottom view of the contaminated water inlet distribution network, at the bottom of the pilot unit.

3.2.2. Experimental procedure

At the first experiment (namely R), 160 mg BPA were added in the external tank at time zero, corresponding to an initial BPA concentration of 2,667 μg L^{-1} in the external tank (Table 6). Volumetric flow rate, was measured at 6 L h^{-1}, corresponding to HRT of 1.77 d. Experiment lasted 35 d and artificially contaminated water (simulating contaminated groundwater plume) was collected in daily basis or every two days from the surface of the pilot, just before entering the external reservoir. Samples of 50 ml were taken for HPLC analysis after passing through a membrane filter of pore diameter of 0.45 μm. HPLC analysis procedure is described in detail in the section "Methods".

Table 6. Experimental design: treatments tested in the rhizodegradation pilot.

Symbol	Description	Date	Flow rate (L h^{-1})	HRT (d)	BPA mass added (mg)	Nominal influent concentration (mg L^{-1})
R	Nominal conditions	August '12	6.0	1.77	160	2.67
Q	Nominal conditions	July '13	7.2	1.47	160	2.67
2Q	Increased flow rate	September '13	17.9	0.59	160	2.67
HC	Increased concentration	November '13	17.9	0.59	5005	83.42

The next phase of the experimental procedure, comprised of three runs (namely Q, 2Q, HC) and started with approximately a year's gap from the first. In the first run (namely Q), flow rate was 7.23 L h^{-1} (HRT = 1.47 d) and for the rest two it was increased at 17.9 L h^{-1} (HRT = 0.59 d, Table 6) and was used to compare these two experimental runs, in terms of plant growth and reliability of the system. It is worth noting that repetition of runs R and 2Q were conducted, which confirmed the repeatability of the process performance (data not showed).

In order to estimate the robustness of the rhizodegradation system, in addition to the experimental runs with high volumetric flow rate which have been mentioned before, another experimental run, namely "HC" was conducted which realizes the scenario of a great deviation of the process input (high BPA concentration and volumetric inflow rate) compared to the nominal case.

In technical literature, robustness is used as a requirement for good structural design in order to characterize a structure and its parts to maintain the amount of failure or deterioration within acceptable limits in relation to the cause design (Fink et al., 2009). In our case the two most important characteristics for rhizodegradation of the polluted aquifer (soil concentration and volumetric inlet flow), were set at extreme values to test if it cause adverse effects on system's performance.

Hence, robustness and resilience of the system was tested, firstly by doubling its flow rate, as previously noted and (with the nominal input of 160 mg to the external vessel), and secondly by increasing the amount of BPA added to the system at time zero, well beyond environmental values (5000 mg total BPA addition). These values corresponded to inlet concentrations of 2.67 mg L^{-1} and 83.42 mg L^{-1} respectively. A last treatment was designed in order to test the effect of biostimulation, with the addition of organic acids five times the real concentration, as measured in the pilot in preliminary experiments. This run is not presented in this thesis as it is described in detail in the study of Syranidou et al. (2017).

Hydraulic retention (or residence) time was determined using the following equation:

$$\mathrm{HRT} = \frac{\mathrm{V}_L}{\mathrm{Q}} \qquad \text{(Equation 14)}$$

where HRT is given in days, V_L the water volume inside the pilot (corresponded to the maximum holding water capacity of soil (0.255 m^3), and Q the flow rate of the contaminated water in $m^3\,d^{-1}$.

Soil used for the experiment was a mixture of soil collected from the field in Akrotiri (Chania, GR) and beach sand (prewashed), in order to increase the permeability and have rapid infiltration of the water. Soil samples were collected for analysis, prior to the beginning of the

experiment and Characterization of the soil after particle-size distribution analysis showed that it was a poorly graded sand with silt with 88.6% sand, 6.0% silt and 5.4% clay. Analysis obtain an alkaline soil, with moderate to good shear strength, very low to medium compressibility and medium workability. Additional information about physicochemical properties and nutrient analysis are shown in Table 7.

Table 7. Content of nutrients in soil (left) and physicochemical characteristics of the soil in the Rhizodegradation pilot (right).

Parameter	Value
pH	8.68
Organic matter (%)	0.5
Total $CaCO_3$ (%)	18.1
Cation Exchange Capacity (me/100 g)	4.49

Parameter	Value ($mg\ kg^{-1}$)
NO_3-N	4.9
P	8.0
K	34.4
Mg	210.0
Mn	7.99
Zn	1.17
Cu	1.94
B	0.31

3.3. Small scale HSF-CW for secondary treated municipal wastewater

Five young *J. acutus* plants were collected from Souda Bay (Chania, Greece), transplanted to the wetland mesocosm and irrigated with wastewater for 4 weeks to allow acclimatization to experimental conditions. The system was set up in the wastewater treatment plant (WWTP) of the city of Chania (35° 32' 19.666" N 24° 3' 7.330" E). A schematic diagram of the wetland system is given in Fig. 9. CW mesocosm, made from stainless steel (2 m x 0.5 m x 0.5 m), was filled with fine gravel (size 0.8-1.25 cm) of total volume of 0.4 m^3. Gravel is widely used as substrate due to its good hydraulic conductivity, coarse structure and the fact that it is not clogged easily (Upadhyay et al., 2016). Secondary treated wastewater, before entering into the wetland, was collected in a polyethylene settling tank of 200 L with the assistance of an electric submersible pump. An electric float switch controlled the operation of the pump. The mesocosm was supplied with wastewater from the settling tank in a continuous mode by means of peristaltic pumping.

A stock solution of a mixture of the target compounds was diluted in a stainless steel tank of 50 L and kept homogenized with the use of a power steering pump. A second peristaltic pump was used for the injection of the organic compounds to the CW system before entering the wetland system, allowing flow rates from 0.25 – 0.5 L h^{-1}. Refill of the spike tank was made every 4 days when HRT was 2 days, or every 2.5 days when the HRT was 1 day. Spiking was taking place by adding i) 25 ml of BPA stock in 50% ACN : tap water, ii) 500 ml from the stock of CIP in acidified water and iii) 50 ml from the stock of SMX in 50% MeOH, to 30 L tap water. Wastewater was contaminated with the pollutants just before entering the wetland. Total volume of treated wastewater inside the wetland was kept constant at 157 L, corresponding to a level of 10 cm below the gravels surface, due to a pipe from which the effluent overflowed from the bottom of the pilot to the specific height. Meteorological parameters (temperature and relative humidity) were monitored every 3h, by a DT-171 data logger, placed at the bottom of the spike tank.

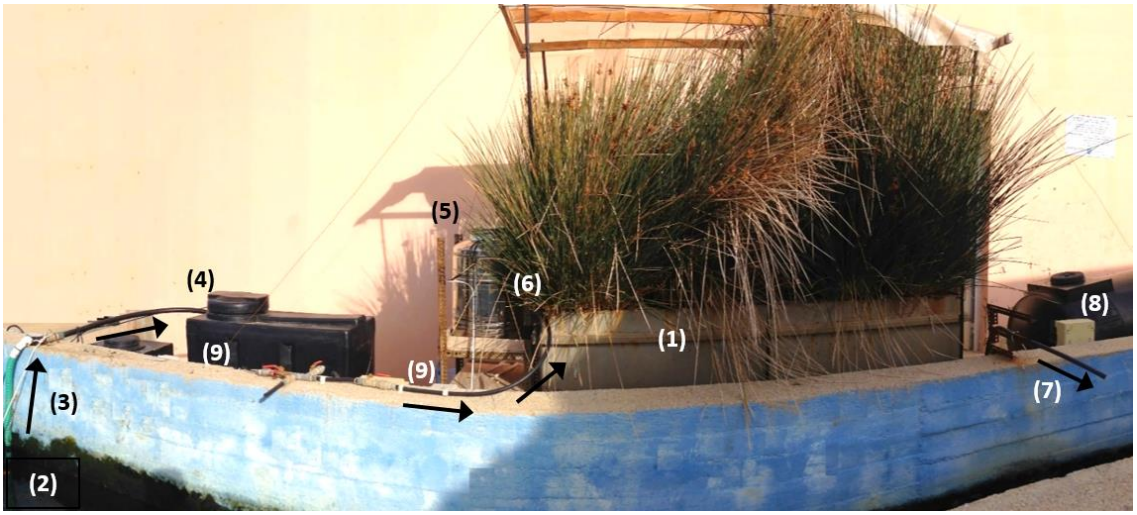


Figure 9. Representation of the horizontal subsurface flow system; (1) horizontal flow constructed wetland, (2) secondary treated wastewater, (3) wastewater inlet to the settling tank, (4) settling tank, (5) organic contaminants tank, (6) wetland input (sampling point), (7) wetland output / treated wastewater overflow (sampling point), (8) electrical panel and (9) peristaltic pumps, placed at the back of the wall.

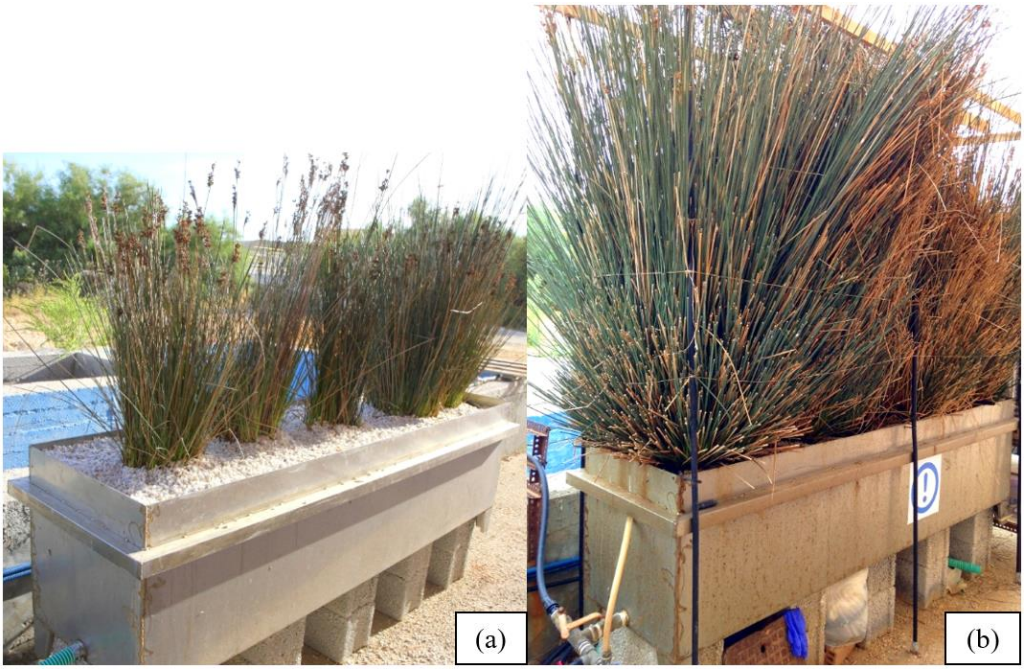


Figure 10. (a): The small-scale HSF constructed wetland in the day of planting (May 2014) and (b): after 2.5 years (October 2016). On the left bottom corner it is shown the overflowing system of treated wastewater.

As far as the first phase of the experimental procedure is concerned, two runs of 50 and 41 days were conducted in summer and autumn 2015, in order to evaluate the efficiency of the system in terms of BPA loading (Table 8). BPA was spiked at a nominal concentration of 5 μg L^{-1} and samples were taken every 4 and 2 days at each run, respectively.

In the next phase of the experimental process, the aim was to study the effect of vegetation, HRT and seasonality on the removal of the specific EOCs by the CW in short term experiments,

of approximately 14 days (Table 8). These were carried out from January to November 2016. Research focused on the investigation of specific parameters, such as the impact of vegetation, HRT and seasonality. The first run was used as a control, without the presence of vegetation. It is important to be mentioned that control run took place in a different wetland with half the size of the vegetated wetland (1 m x 0.5 m x 0.5 m). The rest of the experimental design remained the same as previously described. In this treatment as such as in T treatment, wastewater was spiked with high concentrations of the contaminants, i.e. 100 μg L^{-1} BPA, 1 mg L^{-1} CIP and 5 mg L^{-1} SMX, while HRT was set to 1 day (Table 8).

In HR1 treatment low concentrations of the contaminants were spiked into the wastewater, the same as those of the next treatment (HR2). The following treatment, namely PR, was the only one that used primary treated wastewater. To cope with these circumstances, experimental setup was slightly changed; thus, a polyethylene reservoir of 200 L was used to store the wastewater, before entering the wetland. Refill of the tank with fresh primary treated wastewater was performed every two days. Both HR2 and PR experiments took place during summer (June and July). The last run namely OB, was targeted to investigate the effect of seasonality regarding BPA concentration removal. Hence, experimental design remained the same with HR2 treatment, apart from environmental conditions, since it took place on December. With respect to antibiotics, concentrations where relatively high to obtain easier and low cost chemical analysis.

Table 8. Experimental design: treatments tested and spiked concentrations

Treatment	Time period	HRT (d)	Nominal spiked concentrations at the influent		
			BPA (μg L^{-1})	CIP (mg L^{-1})	SMX (mg L^{-1})
***J. acutus* L1**	3/7 – 21/8/2015	2	5	-	-
***J. acutus* L2**	5/9 – 14/10/2015	2	5	-	-
Control (no plants)	11 – 22/1/2016	1	100	1	5
***J. acutus* T**	7-19/3/2016	1	100	1	5
***J. acutus* HR1**	14 – 28/4/2016	1	5	0.25	0.5
***J. acutus* HR2**	28/5 – 10/6/2016	2	5	0.25	0.5
***J. acutus* PR**	11/7 – 27/7/2016	2	-	-	-
***J. acutus* OB**	4/11 – 28/11/2016	2	5	-	-

Water samples from the influent and the effluent of the wetland were taken on a daily basis for organic contaminants analysis (100 ml sampling volume) and every two or three days for physicochemical properties and nutrients analysis (700 ml sampling volume). It is worth noting that after the last day of each treatment, sampling continued for a week or more, depending on the residual quantities of the compounds measured. Antibiotics and BPA concentrations were determined by HPLC, according to the methods described by Christofilopoulos et al. (2016) and Yiantzi et al. (2010) and presented earlier. All wastewater samples were passed through glass fiber filters of 1 μm, acidified to pH 2.5 (±0.2) and stored refrigerated at 4 ^{o}C, before injected into the HPLC.

Calculations

For the optimal design of a CW and the assessment of system's performance, certain calculations are specified in this section. As a matter of fact, the wetland evapotranspiration that may be more significant for wetland design projects, was evaluated in order to estimate the outlet flow rate (Q_t), the adjusted concentration of the contaminant (C_{out}^{adj}) at the outflow, for the determination both of the actual efficiency in the mass concentration abatement (m_i^{rem}) and the rate of the mass removal of the organic contaminants (r_{out}). During the control experiment the evapotranspiration value (ET) was considered equal to zero. The adjusted concentration at the outflow was calculated by using the equations (15) and (16), according to Lv et al. (2016).

$$\Delta V = \frac{(Qt - V_{out})}{Qt} \qquad \text{(Equation 15)}$$

or

$$\Delta V = \frac{Q_{in-}\, Q_{out}^{adj}}{Q_{in}} \qquad \text{(Equation 16)}$$

where ΔV expresses the losses due to the evapotranspiration, Q_{in} is the actual inlet flow rate in litres per day (L d^{-1}), t is the duration expressed in days (d), V_{out} is the volume in the outlet in liters (L) and Q_{out}^{adj} is the adjusted flow rate in the outflow of the wetland expressed in litres per day (L d^{-1}).

In this respect, according to the equation (3), the adjusted flow rate for the outflow is estimated by the difference of the inlet flow rate (Q_{in}) minus the average hourly evapotranspiration.

$$Q_{out}^{adj} = Q_{in} - ET/24 \qquad \text{(Equation 17)}$$

where ET is the measured evapotranspiration value in litres per day (L d^{-1}).

Therefore, the value for the measured concentration in the outlet was obtained by using the eq. (4):

$$C_{out}^{adj} = (1 - \Delta V)\, C_{out}^{m} \qquad \text{(Equation 18)}$$

where C_{out}^{adj}, C_{out}^{m} is the adjusted and the actual (measured) concentration in the outflow of the CW, respectively (expressed in μg L^{-1} for BPA and mg L^{-1} for both antibiotics).
The percent removal of the organic compounds was defined as follows:

$$Contaminants\ removal\ (\%) = \frac{C_{in} - (1 - \Delta V)\, C_{out}}{C_{in}} x\ 100 \qquad \text{(Equation 19)}$$

where C_{in}, C_{out} are the concentrations in the influent and the effluent, respectively, in μg L^{-1} for BPA and in mg L^{-1} for the antibiotics.

The rate of mass removal of the organic contaminants (r_{out}, in μg h^{-1} or mg h^{-1}, depending on the compound) was calculated by the difference between BPA mass in the influent and the effluent (Equation 20).

$$r_{out} = m_1 - m_2 = Q_{in}\, C_{in} - Q_{out}^{adj}\, C_{out} \qquad \text{(Equation 20)}$$

m_1, m_2 are BPA mass at the influent and the effluent, Q_{in}, Q_{out} are the total influent and effluent flow rates and C_{in}, C_{out} are the concentrations at the influent and the effluent, respectively.

Another indicator that was proved to be equally important for assessing the system's performance, was obtained by combining equations (21), (22) in order to calculate the ratio of the organic compound mass removed, over the input mass (m_p) (Hijosa-Valsero et al., 2010b).

$$m_i^{rem} = m_o^{in} - m_i^{out} \qquad \text{(Equation 21)}$$

$$m_i^{out} = Q_{out\,i}^{adj}\, x\, C_{out\,i}^{meas}\, x\, \Delta t_i \qquad \text{(Equation 22)}$$

$$m_p(\%) = \frac{m_i^{rem}}{m_o^{in}} x\ 100 \qquad \text{(Equation 23)}$$

Where m_i^{rem} is the mass of the organic compound that is removed from the system during the time estimated in milligrams per hour (mg h^{-1}), m_i^{out} the mass of the compound at the same time in milligrams per hour (mg h^{-1})[1], $Q_{out\,i}^{adj}$ the adjusted flow rate at the outlet in liters per hour (L h^{-1}), $C_{out\,i}^{meas}$ the measured concentration of the compound at the effluent of the system in

milligrams per liter (mg L^{-1}) and Δt the time elapsed expressed in hours (h). Considering that in some cases the residence time was 2 days, the Δt_i was doubled for the implementation of m_i^{out}, for the inlet mass to be kept the same with the one calculated with residence time of 1 day.

3.4. CW with halophytes for the degradation of BPA in primary-treated municipal wastewater

3.4.1. Experimental design

The study was conducted in Heraklion, Crete (N 35º,19”; E 25º,10’’) in a pilot plant consisted of two wetlands, a surface flow (SF) CW and a horizontal subsurface flow (HSF) CW working in series (Fig. 11). The system was fed with primary treated municipal wastewater (following primary sedimentation), spiked with the contaminant (mean influent nominal concentration of BPA, 0.27 mg L^{-1}). The HSF system which covered a surface of 45.36 m^2, was planted with four mediterranean wetland halophytic species; namely, *Tamarix parviflora* (5 plants), *Juncus inflexus* (8 plants), *Limoniastrum monopetalum* (8 plants) and *Sarcocornia perennis* (15 plants). Substrate of the HSF CW was a 30 mm gravel layer, with a depth of 45 cm, while two layers of 60 and 100 mm coarse gravel were positioned each in the influent and the effluent side of the wetland. Distribution of wastewater was done with the assistance of a perforated polyethylene 50 mm pipe, which was positioned in the long side of the wetland (Fig. 12). An external reservoir of 75 L was used for the artificial contamination of the influent wastewater with BPA, by means of a dosing pump with a flow rate of 0.035 L h^{-1} (Fig. 13).

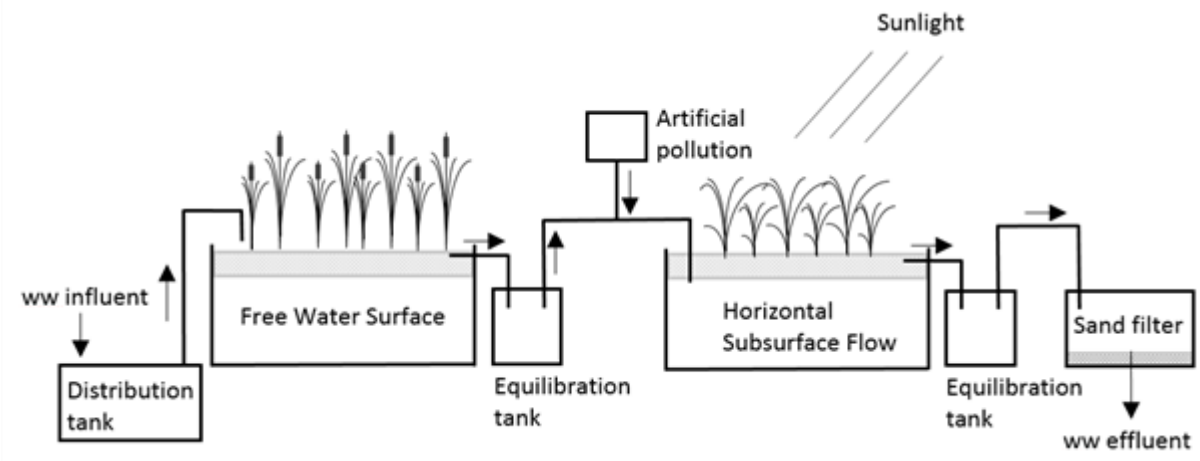


Figure 11. Diagrammatic representation of the pilot system, with the two constructed wetlands in series used in this study

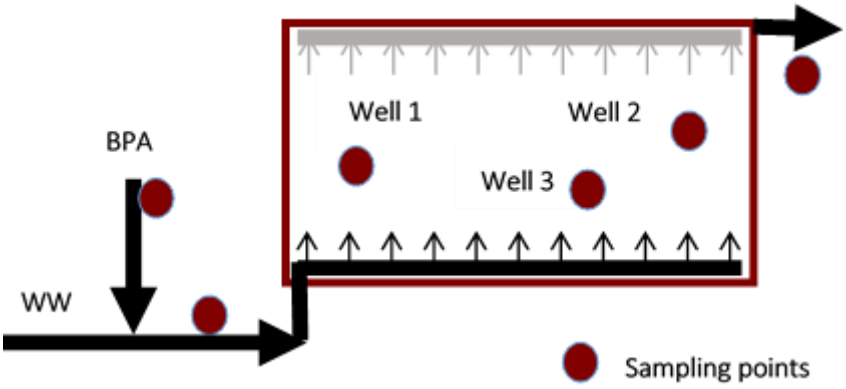


Figure 12. Schematic diagram of the HSF-CW (top view), showing the exact position of three sampling points inside the wetland and the other three in the influent, the effluent and the spike tank. Sampling points are indicated with red dots.

Influent flow rate, controlled by a timer switch, was set to activate the wastewater pump twenty times per day for 2-5 min, so as the total volume of the wastewater fed to the wetland to correspond to the desired HRT (shown in Fig. 11). Therefore, while the overall hydraulic loading was 1.4 m^3 d^{-1}, the actual flowrate was varied to emulate the actual flowrate encountered in a municipal WWTP. This is shown in Fig. 12 below. Equation (14) was used for the determination of HRT, where: V_L = 2.59 m^3 and Q: influent flow rate (m^3 d^{-1}). The input from the WWTP (Q_1=1386 L h^{-1}) was spiked with BPA using a dosing pump (Q_2=2.1 L h^{-1}) in the inlet of the constructed wetland (Q). The BPA reservoir solution was about 100 ppm and hence the nominal input concentration of BPA to the CW was 100 mg L^{-1} × $Q_2/(Q_1+Q_2)$, i.e., 150 μg L^{-1}.

Figure 13. View of the pilot scale HSF-CW (on the left) and the BPA adding set up (on the right).

Three different hydraulic residence times (HRTs) were applied to the system in order to evaluate the dependency of treatment efficiency (COD and micro-pollutant's removal) on HRT

(Table 9). Under nominal conditions (flow rate 1.41 m^3 d^{-1}, corresponding to HRT = 1.84 d) the system ran from 31st May to 6th August 2013. Afterwards, flow rate was switched to double of the nominal (2.88 m^3 d^{-1}) and kept for 18 days (19th August). Flow rate was then switched to approximately half of the nominal, i.e. 0.74 days, corresponding to a flow rate of 0.74 m^3 d^{-1}, until 2nd December. After a period of non-operation due to feed pump failure, where no data were collected, system was restarted at the 25th November with the same HRT, until 11th of December. In this period, CW was bioaugmented in the 3rd December with 15 g of bacteria *Sphingomonas* sp. strain TTNP3, with the assistance of the piping wastewater distribution system, after dilution in 20 L of tap water.

Table 9. Information on operational phases (relating to BPA degradation)

Phases	Treatment description / Conditions
I	Nominal flow HRT = 1.8 d, Q_{BPA} = 2.13 L d^{-1}
II	Double flow HRT = 0.9 d, Q_{BPA} = 4.27 L d^{-1}
III	Half flow HRT = 3.4 d, Q_{BPA} = 1.12 L d^{-1}
IV	Half flow + TTNP3 HRT = 3.4 d, Q_{BPA} = 1.12 L d^{-1}

Removal efficiencies for wastewater parameters were calculated as the percentage change in concentration from influent to effluent using equation (24):

$$Removal\ efficiency = \frac{Ci - Co}{Ci}\ x\ 100\% \quad \text{(Equation 24)}$$

Ci: concentration in the influent, C_o: concentration in the effluent.

Sampling method

Samples for BPA analysis were taken from the influent, the effluent, the spike tank as well as from three sampling wells inside the wetland every 1-3 days. Samples were filtered (0.45 μm, Whatman glass fiber) and kept in 4 ºC. Regarding water quality characteristics, samples were taken from the influent and the effluent. Analysis included pH, electrical conductivity, COD, total nitrogen, total phosphorus, boron, nitrate and ammonium, according to the methods described in the related section of this thesis.

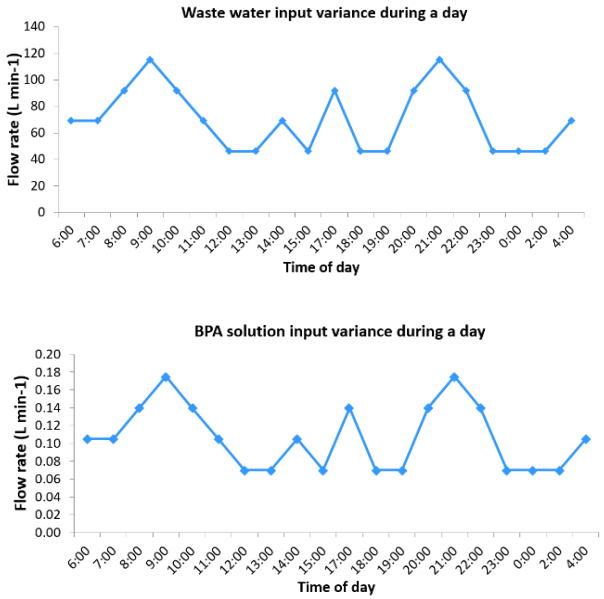


Figure 14. Daily variation in the inlet flowrate of wastewater (above) and the contaminant's flowrate to the constructed wetland (below).

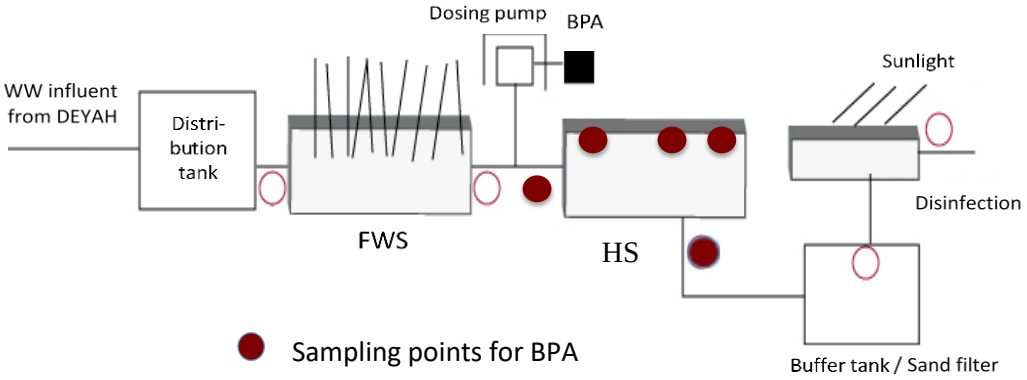


Figure 15. Schematic diagram of the large scale HSF-CW (side view). Sampling points are indicated with red dots.

CHAPTER 4. MATERIALS AND METHODS

4.1.1. Chemicals and solvents

Separation and quantification of the organic compounds was carried out using a high performance liquid chromatography (HPLC) system, manufactured by Shimadzu (Shimadzu Corporation, Kyoto, Japan), equipped with LC-10AD VP solvent delivery modules, SPD-M10 AVP Diode Array Detector (DAD), RF-10AXL Fluorescence Detector and SIL-10AD VP autosampler. Bisphenol A (BPA) (97% purity) and acetonitrile (99.99% purity, HPLC gradient grade) were purchased from Sigma–Aldrich (Germany). Ciprofloxacin (CIP) (≥98.0%), HPLC grade) and sulfamethoxazole (SMX) were purchased from Fluka (Germany). Ethyl acetate (99.9%, HPLC grade), methanol (≥99.9%, GC) and acetone (HPLC, ≥99.9%), used for the extraction of BPA from soil, were purchased from Sigma–Aldrich (USA). Deionized water was produced on a Barnstead/Thermolyne Easypure RF purification system (Dubuque, IO, USA). All heavy metals were purchased from Sigma–Aldrich (USA) and added in the nutrient solution as $K_2Cr_2O_7$, $NiCl_2 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_20$ and $Cd(NO_3)_2 \cdot 4H_2O$.

4.1.2. Organic compounds analysis

Separation of BPA was performed on a Nucleosil 100-5 C-18 (250 mm × 4.6 mm × 5 μm; Macherey-Nagel, Duren, Germany) column at 27 ^oC. The mobile phase was ACN :water (50:50), the excitation and emission wavelengths of the Fluorescence Detector (FLD) were set at 277 and 300 nm respectively and analysis time was 15 min. Extraction of BPA from samples of low BPA concentrations (up to 0.5 μg L^{-1}), was carried out employing the vortex-assisted liquid-liquid microextraction protocol that has been developed in our lab by Yiantzi et al. (2010)

An Alltech Prevail™ Organic Acid 5u (250 mm × 4.6 mm × 5 μm; Deerfield, IL, USA) was used to separate the antibiotics CIP and SMX at 27 ^oC. The mobile phase consisted of 0.02 M ortho-phosphoric acid buffer (pH 2.45) and ACN (50:50) and the flow rate was maintained at 1.0 $mL.min^{-1}$. The column effluent was monitored for SMX on a UV detector set at 266 nm, while excitation wavelength of 278 nm and emission of 445 nm was set in RF detector for CIP detection. Samples were direct injected into the rheodyne valve.

The experimental results of the HSF-CW of Chania, were statistically evaluated using the SPSS Statistics 20 package (Chicago, IL, USA). Paired sample t-test, Wilcoxon pair test, independent t-test and Mann-Whitney U test, were used according to the circumstances (dependent or independent samples, normally distribution or not), in order to evaluate the statistical significance between the results (significance level was set at $p < 0.05$).

4.1.3. BPA extraction from soil

Soil extraction from soil was conducted after modifications in methods reported from Ying et al. (2003) and Xu et al. (2008). Preliminary experiments were conducted in order to determine the most efficient technique for spiking and extraction BPA from soil samples. Method development required testing of different solvents (ethyl acetate, acetone and acetonitrile), soil per solvent rates and extraction techniques (stirring, vortex, sonication, Soxhlet extraction) These preliminary tests revealed the procedure we followed for the extraction. Consequently, soil was dried at 80 ^{o}C for 48 hours and determination of BPA remaining in soil was carried out according to the following procedure: 5 mL of acetone-ethyl acetate (1:1, v/v) were added into 25-mL glass centrifuge tube containing 5 g of soil. The tube was ultra-sonicated for 15 min, centrifuged at 3500 g for 5 min and supernatant was decanted. The soil was extracted three more times, and the supernatants were combined and were nitrogen evaporated to dryness. The extract was re-dissolved in 1 mL of MeOH and direct injected in HPLC. Quantification of the compound was made due to the isocratic method using 50% ACN, described in the previous paragraph.

4.1.4. Heavy metals analysis

Analysis of aqueous samples was carried out through Inductively Coupled Plasma Mass Spectrometry (ICP-MS 7500cx coupled with Autosampler Series 3000, both of Agilent Technologies).

All solid samples were analyzed for the quantitative determination of heavy metals, using the above mentioned device, following digestion in a microwave digestor (Multiwave 3000 by Anton Paar, Graz, Austria). Liquid digestion was performed using a modification of the standard operating procedure of EPA 3051a (USEPA, 1995). In particular, 0.2 g of dried sample was weighed and digested with 9 mL HNO_3, (>69%, Sigma-Aldrich). Subsequently, digested samples were diluted with ultrapure water and centrifuged. Supernatant was separated, filtered (0.45 μm, Whatman), diluted at 1:10 (v/v) with ultrapure water and analyzed by ICP-MS.

4.1.5. Chlorophyll measurements

Plants were assessed for their chlorophyll content following the protocol method described Harborne (1984) Leaf samples (0.2 g) were collected and homogenized with 10 mL 80% acetone. Subsequently, they were centrifuged twice at 16000 rcf for 1 minute and the absorbance of the supernatant was measured at 663 and 646 nm with the use of a UV-VIS spectrometer for the determination of chlorophyll a, chlorophyll b and total chlorophyll concentrations.

4.1.6. Guaiacol peroxidase activity and protein content

Fresh plant material (1 g leaf and root) was crushed with liquid N_2 and homogenized with 10 mL 0.05 M phosphate buffer (pH 5.8) with a mortar and a pestle. The homogenate was passed through a 4-layer cheesecloth and the filtrate was centrifuged for 20 minutes at 16000 rcf and 4 ^{o}C. The supernatant was centrifuged again at 16000 rcf and 4 ^{o}C for 5 minutes. The total protein content of the plant tissues was measured according to the modified Lowry Protein Assay Kit protocol. Activity of guaiacol peroxidase (GPX) was determined using the method of Erdelský and Frič (1979) at the same extraction mixture as the proteins. Briefly, a reaction mixture containing 0.05 M phosphate buffer (pH 5.8), 15mg mL^{-1}. Guaiacol, a suitable aliquot of enzyme and 1% H_2O_2 was prepared and the absorbance was monitored at 470 nm with the help of a UV-Vis spectrometer for 3 minutes. The activity was measured by the increase in absorbance due to the oxidation of guaiacol and the enzyme activity unit was expressed as the change in absorbance per minute (ΔA 470 min^{-1}). The specific activity of enzyme is expressed in terms of units per milligram of extracted proteins. Each sample was measured in duplicate.

4.1.7. Catalase activity

Fresh plant samples (0.5 g leaf and root) were collected, crushed with liquid N_2 and homogenized with 100 mM potassium phosphate buffer (pH=7) containing 0.1 mM EDTA and 1% polyvinylpyrrolidone (w/v) at 4 °C for the extract preparation according to the method of Mishra et al. (Mishra et al., 2006) The homogenate was filtered through four layers of cheesecloth and centrifuged at 15.000 rcf for 15 minutes at 4 °C. Supernatant was used to measure catalase (CAT) activity. CAT activity was assayed by the method described by Aebi (Aebi H, 1984). The reaction mixture (total volume 3 mL) contained 50 mM sodium phosphate buffer (pH 7.0), 30 mM H_2O_2 and a suitable aliquot of supernatant enzyme. Catalase activity was measured as the decrease in absorbance at 240 nm for 3 minutes, using the absorption coefficient of 43.6 M^{-1} cm^{-1}. Enzyme activity was expressed as mmoles of H_2O_2 degraded min^{-1} g^{-1} fresh weight (fw). Each sample was measured in duplicate.

Statistical analysis of chlorophyll content, GPX, protein content and CAT was performed with t-test, using the Add-In statistical package in Microsoft Excel (significance level set at $p<0.05$).

4.1.8. Wastewater quality parameters

Analysis of total suspended solids (TSS), chemical oxygen demand (COD), biological oxygen demand (BOD_5) and total organic carbon (TOC) were conducted according to Apha standard methods (Apha 1985). Total carbon (TC), inorganic carbon (IC) and TN were measured in Analytik Jena's "multi N/C 2100S" from unfiltrated samples. TOC was determined as the numerical difference between TC and IC. Electrical conductivity (EC) and pH were measured by a Hach HQ40d multi parameter meter. All the above were measured in Biochemical

Engineering and Environmental Biotechnology Laboratory at TUC. Statistical analysis was performed using the SPSS Statistics 20 package (Chicago, IL, USA), as described in the results section (Chapter 5).

Regarding the analysis of the large-scale HSF-CW system, COD, TN, TP, nitrate, ammonium and boron, were analyzed using standard test kits (Hach-Lange GmbH) and DR 2800 spectrophotometer (Hach-Lange GmbH). In addition, pH was measured with a pH-meter (WTW, 3110) and EC with a conductivity meter (Hanna, 8333) in accordance with Standard Methods (APHA, 1995). Unfiltered BOD_5 was analyzed using WTW OxiTop meters. These analyses were performed by Solid Waste & Wastewater Management Laboratory in TEI -Crete.

Statistical analysis of wastewater quality parameters of the of the large-scale HSF-CW system

The data were analyzed by one-way analysis of variance (ANOVA) to compare the variation of pH, EC, COD, BOD, total nitrogen, nitrate-N, ammonium-N, total phosphorus and boron concentration in the influent and the effluent. A Tukey test was performed to detect the statistical significance of differences ($p<0.05$) between means. The statistical analyses were carried out with MicroCal Origin 7.0 (OriginLab).

CHAPTER 5. RESULTS AND DISCUSSION

5.1. Small- scale experiments

5.1.1. Pot experiments in soil substrate

Polyethylene bags (PEBs) with J. acutus in growth chambers

This study took place in growth chambers in order to obtain favorable environmental conditions (light intensity and photoperiod) and repeatability of the procedure. Pots were supplemented with 5.45 mg BPA and the efficiency of the planted treatments in comparison to the controls, after 6 days of treatment, was assessed. BPA mass in both aqueous and soil phase was measured and total removal of the compound estimated, as described in the experimental design. The results indicated remarkable uniformity. Mass removal in the aqueous phase was decreased by 76% in vegetated treatments and 10% in controls (days 1-6), when BPA mass in the 1st day was measured at 0.50 mg and 0.55 mg, respectively. In soil phase, attenuation of 76% of 2.42 mg was calculated for vegetated and 22% of 2.08 mg for control treatments. With regard to the total BPA mass in the system, removals of 76% and 20% were measured in the case of *Juncus* vegetation (2.66 mg at day 1) and non-planted treatments (2.97 mg at day 1), respectively.

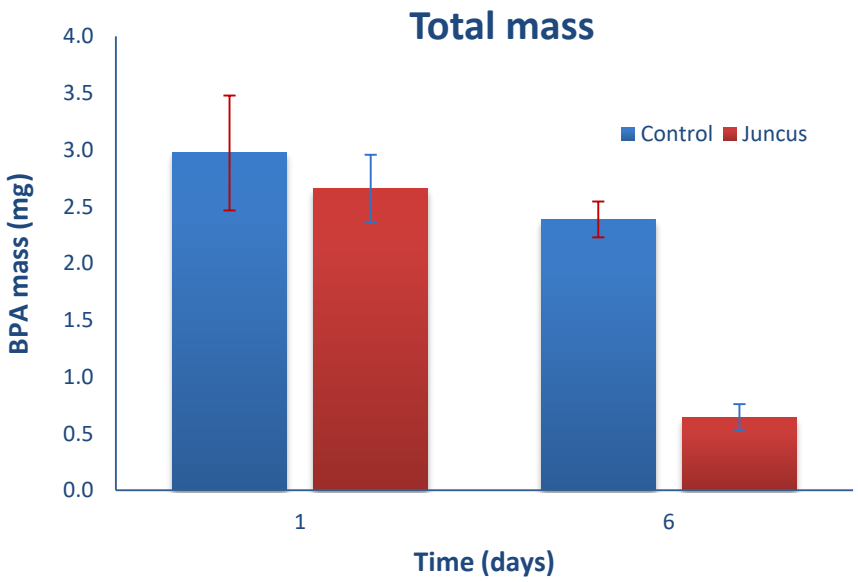


Figure 16. Total BPA mass (in the soil and the aqueous phase) remaining for control and planted experimental treatments at the PEBs remediation procedure.

Taking into account nominal initial BPA mass added in each replicate at time zero, mass removal for non-planted treatments was found to be 56% and for the planted ones 88% (days 0–6). The effect of the presence of plants on the removal of BPA, as expressed by apparent degradation rate constant (k'), was obtained using equations (6) and (7), and they are presented in Fig.17 and summarized in Table 11. It is important to mention that graph in Fig. 17 is derived by the concentrations measured only in the aqueous phase, where degradation is attained.

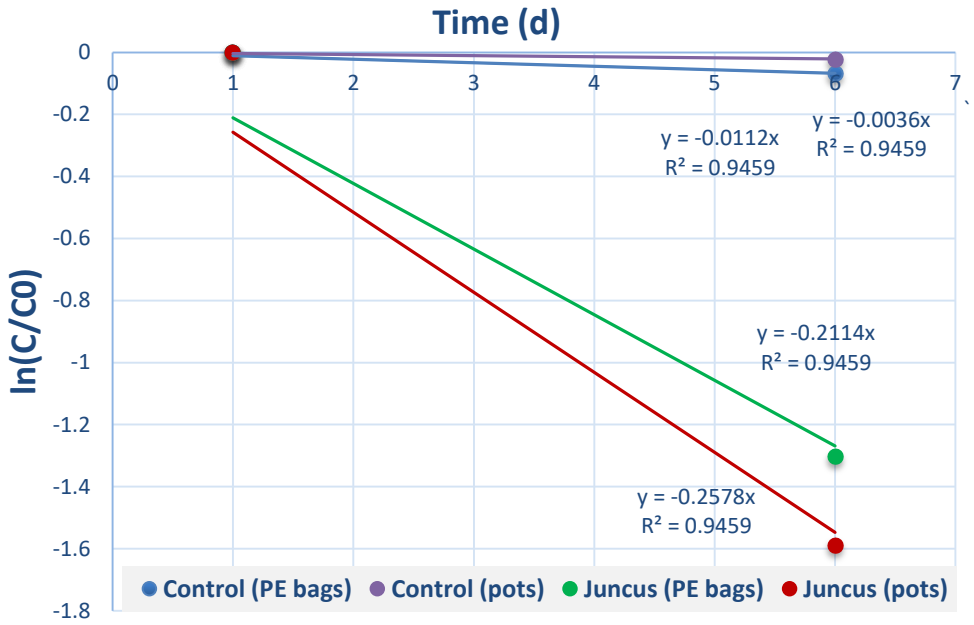


Figure 17. Apparent first order degradation rate in the aqueous phase, in pot and PEBs experiments (days 1-6).

Pot experiment with *J. acutus* in growth chamber

Experimental design in this study was changed in order to give more space for the root system to grow. This was achieved by using ceramic pots of 1.4 L total volume, equipped with a drain valve system that allowed recirculation of the contaminated water, when needed. Handling space requirements and specific chamber dimensions led us to use less number of replicates. Results of the analysis in terms of the total BPA mass remaining in the system after six days of treatment, are shown in Fig. 18.

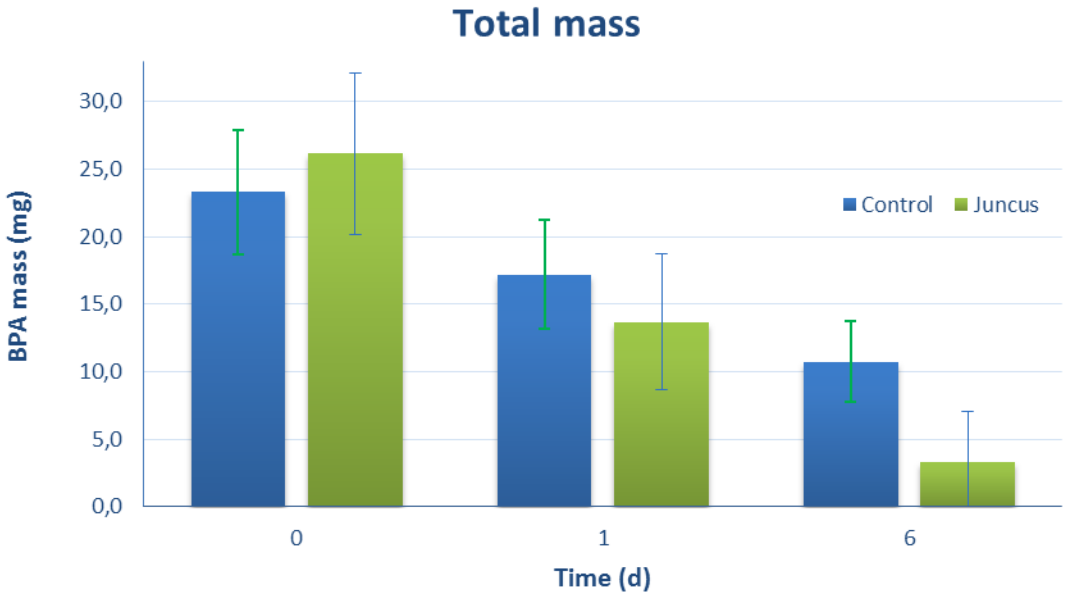


Figure 18. Total BPA mass remaining in the pots system, during the experimental period in growth chamber.

Total BPA mass removal was decreased by 54% in control and 87% in planted treatments respectively, after six days of treatment (days 0 – 6, Fig. 18). It is worth mentioning that recirculation of the initial solution was taken place after BPA spike and before every sampling. However, a noticeable variation of the measured BPA mass among the two treatments was obtained at day zero (3 h of treatment), 23.3 mg in controls and 26.2 mg in vegetated pots. In the case of soil phase, where it is likely that adsorption of this moderately high hydrophobic compound to the extended outer roots may be occurred (Ávila et al., 2010)(Wu et al., 2013), results are not in conformity with this theory, possibly due to the limited time given for the process to be carried out. However, in contrast with PEBs experiment, data displayed large oscillations with concentrations bouncing from high values down to approximate the analytical detection limit of 0.5 μg L^{-1}.

Table 10. Percentage removal efficiency of BPA mass for growth chamber experiments, given separately for aqueous, soil phase and overall (days 1-6).

	PEBs			Pots		
(%)	**aqueous**	**soil**	**total**	**aqueous**	**soil**	**total**
Control	10	22	20	7	40	38
Juncus acutus	76	76	76	63	78	76

Overall, results on the analysis of BPA demonstrated that in the present of vegetation, rhizosphere facilitated attenuation of the compound (Table 10). Based on the afore-mentioned results, *J. acutus* is presented as a helophytic species with a potential for application and perspectives of using in contaminated groundwater remediation.

***T. parviflora* and *J. acutus* in pot system under non-controlled conditions**

In this experiment, *Tamarix parviflora* was additionally investigated and its efficiency in the removal of BPA was compared with *J. acutus* and control treatments. Observation of Fig. 19 reveals the attenuation of the compound in both planted and non-planted mesocosms over the experimental period. Attenuation of BPA in the absence of plants was obvious by the removal of 66% of the total BPA in control treatments. Halophytes *J. acutus* and *T. parviflora* resulted in 94% and 90% respectively, showing a significant contribution in the remediation procedure. As previously mentioned, in order to achieve better homogenization of the solution, total water was recirculated twice from the bottom to the surface of each pot, prior to each sampling.

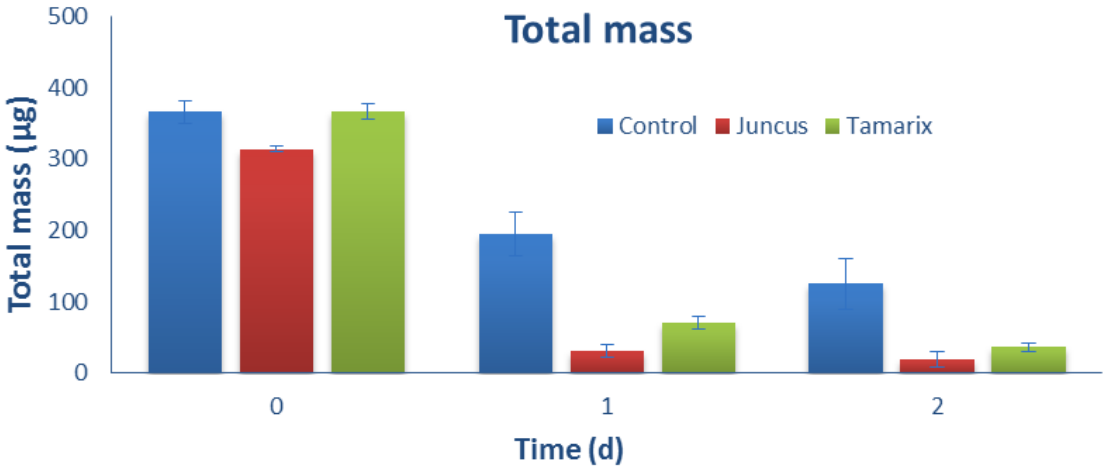


Figure 19. Estimated BPA mass remaining in the soil during the second run in pot experiments, under non-controlled conditions

Notwithstanding, the initial BPA concentration varied among the three. In the case of total BPA mass at day 0 (Fig. 19), this was attributed to BPA residues remaining in the soil from the previous experimental run, that was counted in the initial mass calculations. Furthermore, BPA degradation rates were estimated by Least Squares calculations, with m_{soil} being 1.17 kg and the results were as shown in table 11. According to the results, BPA degradation rate is about two to three times higher in the presence of plants. Regarding distribution of the compound to the soil and the aqueous phase as expressed by K_{SD} coefficient, it was ranged between 5.3 and 10.6 L kg^{-1} for treatments tested.

Table 11. BPA degradation rates as calculated for the pot experiment. The ranged values of control and *J. acutus* groups refer to the initial concentrations of 0.15 and 0.3 mg L^{-1}, respectively

Treatment	Degradation rate (mg BPA kg^{-1} soil h^{-1})
Control (no plant)	0.71 to 0.98 10^{-2}
T. parviflora	1.94 10^{-2}
J. acutus	2.05 to 2.47 10^{-2}

What is more, first order degradation rate constant (k') when assuming rapid thermodynamic equilibrium, was calculated for planted and non-planted treatments using equations (6) and (7) (Fig. 20). Results reveal the contribution of both species on BPA removal while natural attenuation in the absence of plants was kept in low levels, corresponded to k' values of 0.5 and 0.3 d^{-1}. Moreover, the highest removal capacity was not a privilege of a single plant species. However, it should be taken into account that in the first run with *J. acutus* and the corresponding control group, 0.15 mg of BPA was spiked in each pot, while in both runs of *T. parviflora* the initial BPA mass was 0.30 mg.

Table 12. BPA degradation rate constants and apparent rate constants in aqueous phase under thermodynamic equilibrium, for all the experiments. Experimental period was 2 d in glass house experiments and 6 d in growth chamber.

	Non-controlled conditions				**Growth chamber**	
	***J. acutus* pots**		***T. parviflora* pots**		***J. acutus* pots**	***J. acutus* PEB**
	run 1	*run 2*	*run 1*	*run 2*		
Nominal spiked BPA mass (mg)	0.15	0.30	0.30	0.30	30	5.45
k' non-planted (d^{-1})	0.085	0.004	0.469	0.280	0.004	0.011
k' planted (d^{-1})	1.248	1.013	0.914	1.375	0.258	0.211

In a parallel research using the same experimental design, the presence of BPA enhanced the production of low molecular weight organic acids from the halophytes. Organic acids pattern changed in the presence of BPA as the days of treatment progressed, while no organic acids

were detected in the pots with polluted soil (without plants) and pots with the halophyte *J. acutus* in non-contaminated soil (Syranidou et al., 2017).

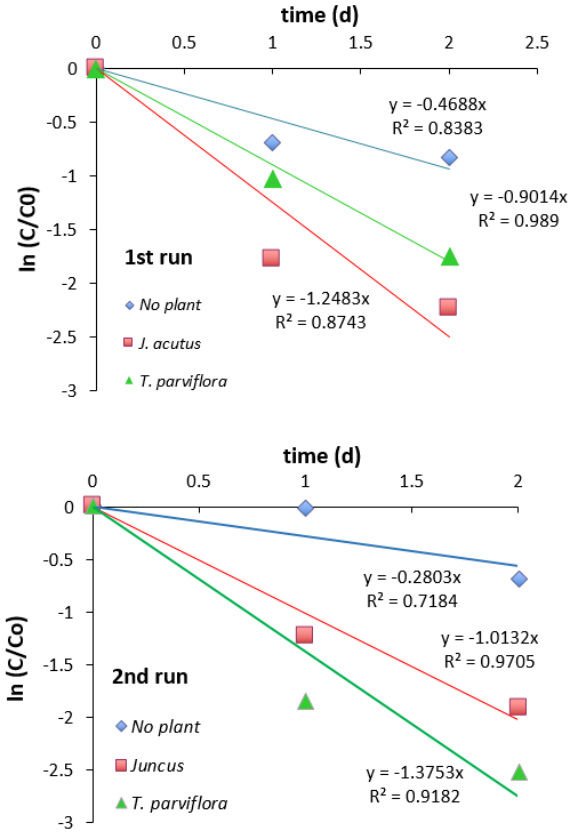


Figure 20. Apparent first order degradation rate in pot experiments conducted under non-controlled conditions

As observed from the Fig. 20, apparent degradation rate constant of BPA was found greater when the initial concentration was lower. In particular, it was found an order of magnitude higher in concentrations of 0.15-0.30 mg of BPA spiked, than in high concentrations (30 mg). In this study due to the small size of the pots and the frequent recirculation, it is supported that aerobic pathways prevailed.

Concluding remarks

Literature on small-scale phytoremediation studies dealing with EDCs removal and particularly BPA, is mainly engaged with plant cell suspension cultures (Saiyood et al., 2010) or plant enzymes (Matsui et al., 2011). In the study of Imai et al. (Imai et al., 2007), 50 μM of BPA were totally removed by the *Portulaca oleracea* in water cultures, within 24 h. The same garden plant was also tested in a hydroponic experiment focused on elucidating the metabolic pathway of the contaminant (Watanabe et al., 2012), while Peroxidases are reported as the primal enzymes responsible for the degradation of the disruptor in a key study with aquatic plants (Reis

et al., 2014). In a more recent and integrated approach to this issue proposed by Syranidou et al. (2017), endophytic community of *J. acutus* was found highly enriched with BPA tolerant strains and potential degraders, giving promises for future applications. However, to the author's knowledge, information concerning the removal of BPA, in small scale soil experiments with the specific helophytes, are not compiled in a public report. Thus, literature review on the removal of BPA is provided in the following section (paragraph 5.1.2), based on hydroponic treatments. However, the present work was performed in soil substrate, in which convoluted soil-microbe interactions prevail, rendering it the most complicated biomaterial in the planet (Young and Crawford, 2004). Consequently, treatment efficiency in soil-plant systems should be thoroughly investigated to reveal the metabolic pathways and the specific mechanisms involved.

Overall, these primary results suggest that *J. acutus* responded satisfactorily on BPA treatment. Besides, due to several other parameters, *J. acutus* was chosen for further insight into phytoremediation research targeting to phytoremediation purposes. *Juncus* plants responded faster and better in propagation in contrast with *T. parviflora*. In fact, multiplication of *J. acutus* via rhizome cuttings, proved to be an easy and very rapid technique that allows the production of plants that require less time to grow and offers uniformity of the replicates. Besides, vegetative propagation skips the risky and time consuming stage of seedling, in which a percentage of plants die. However, care must be taken in order to avoid parent plants to be stressed and lose vigor, due to severe rhizomes cutting. Based on these data and the capacity of *J. acutus* to be used under the saturated conditions of a constructed wetland, this species was chosen to be more extensively investigated in a small CW system experiment (paragraph 3.3)

5.1.2. Small- scale experiment in hydroponic cultures

Organic contaminants

BPA removal

Average overall removal efficiencies of BPA were unanswerable high (Fig. 21). Concentration in treatment E, spiked with 1 μg L^{-1}, could not be detected after seven days, under the analytical conditions used in the experiment. In the case of treatment F, spiked with 0.5 mg L^{-1}, removal efficiency of 96% was observed after 28 days. Approximately equal high was the removal percentage in treatment G, where 98% of the 50 mg L^{-1} initially added in the nutrient were removed. In the absence of plants, where controls exposed in a mixture of contaminants, a 4% reduction of the initial concentration of BPA (0.5 mg L^{-1}) was observed.

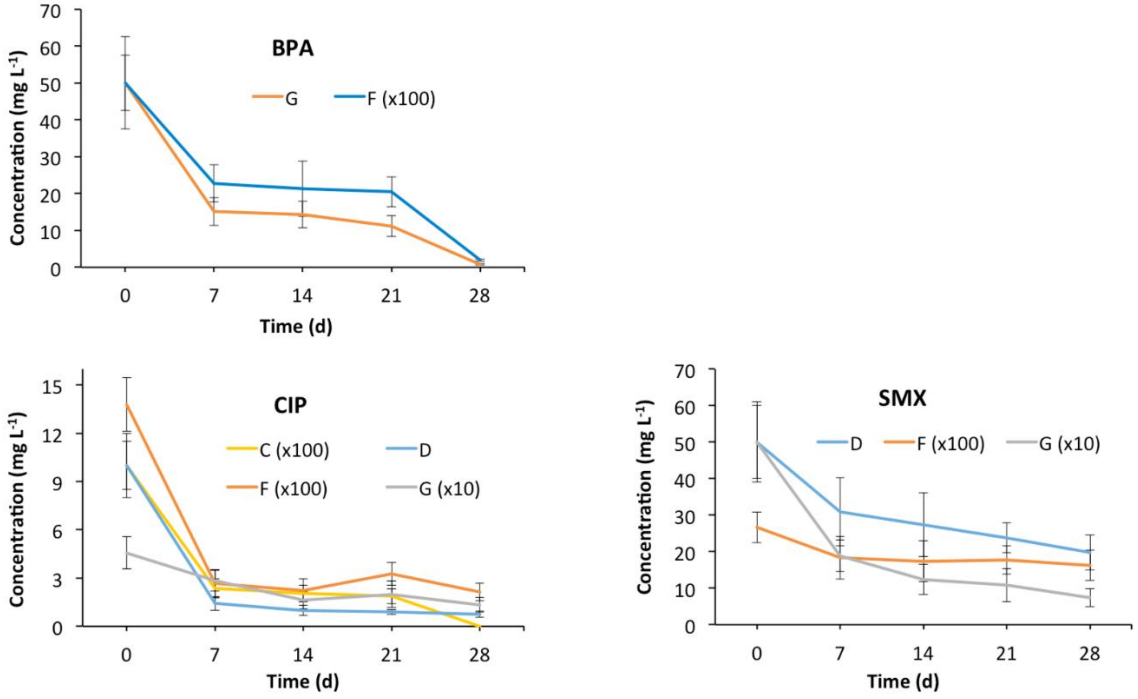


Figure 21. Removal of BPA, CIP and SMX over the experimental period, measured in the nutrient solution every 7 days. Values shown are means (n=7) with standard error bars. Values of treatment C in the case of CIP and values of treatment F for all the tested compounds are multiplied by 100 the real concentration. Values of treatment G are multiplied by 10 for both antibiotics.

In a recent publication, Toro-Velez et al. (2016) examined the efficiency of different HSSF-CWs planted with *Phragmites australis* and *Heliconia psitacorum* for the removal of BPA and nonylphenol (NP) from municipal wastewater. Regarding BPA, removal efficiencies of 73.3% and 70.2% of the 8.8 μg L^{-1} in the influent, were found for the two species respectively, in comparison to 62.2% for the control CW. On the other hand, under hydroponic conditions,

Dracaena sanderiana removed 82.7% of the 20 μM of the compound initially added, with respect to 73.9% of *Dracaena fragrans*, notwithstanding, the first demonstrated a significantly higher uptake capacity.(Saiyood et al., 2010) In a hydroponic experiment of Dodgen et al. (Dodgen et al., 2013), BPA at initial concentration of 46.4 ng L^{-1} show the highest dissipation after 21 days, among the four PPCPs tested (69.1% with lettuce and 88.4% with collards). The greater accumulation of the compound was found in tissues of collard (66.5% of the initial). Moreover, in another hydroponic study using *Ipomoea aquatic*, initial concentration of 5 mg L^{-1} was totally removed after 7 days (Noureddin et al., 2004). Thus, in accordance with the literature, BPA is shown to be easily removable, whereas plant species is a factor that affect the level of efficiency (Dodgen et al., 2013).

Antibiotics removal

Chemical analysis was performed in nutrient solution to determine antibiotics concentration remaining in the aqueous phase. Treatments exposed in very low concentrations of 1 μg L^{-1} and 5 μg L^{-1} of CIP and SMX respectively, were not taken into consideration regarding removal efficiency, since the quantification limit could not allow this analysis, due to the small sampling volume. The meaning of this treatment was the evaluation of physiological response of *J. acutus* under environmentally relevant levels of contamination. Regarding higher concentrations, removal efficiencies of CIP achieved in treatments fortified only with antibiotics reached 95% (Fig. 21). In particular, for CIP concentrations of 0.1 mg L^{-1} and 10 mg L^{-1} the removal was 95% and 92%, respectively. In the case of mixed contamination (treatments F and G), CIP reduction was slightly lower, reaching 84% and 71% respectively. Controls without plantation showed reductions of 59% and 54% of the initial 0.1 mg L^{-1}, where only antibiotics were added and as a part of mixed contamination, respectively. These reductions may occurred due to mechanisms such as adsorption or microbial degradation, as reported for other fluoroquinolone antibiotics (Carvalho et al., 2013). Similarly, 61% SMX removal was achieved in treatment D, initially spiked at the highest value of 50 mg L^{-1}. In the presence of BPA and metals, the removal reached approximately 87% for both initial concentrations of 0.5 mg L^{-1} and 5 mg L^{-1} (treatments F and G). Lower removal in treatment D may attributed to the fact that concentration of the compound was an order of magnitude higher than in treatments exposed in mixed contamination, indicated that the plant might not withstand these conditions. Where no metals or BPA were spiked in the nutrient, 0.5 mg L^{-1} of SMX had been totally removed by day 14. In controls without plants, SMX removal of 14% and 4% was observed, when 0.5 mg L^{-1} of the compound added in the absence or as a mixture with metals and BPA, respectively.

Removal of the above mentioned antibiotics with the assistance of plantation has been mainly studied under CWs experiments, focusing also on the mechanisms involved, however information is still limited (Li et al., 2014; Zhang et al., 2014). SMX is characterized as readily

removed by CWs acting as a secondary treatment, showing in the case of free water surface CW systems, efficiency from 59% (influent concentration was 227 μg L^{-1}) to 92% (influent concentration was 13.8 μg L^{-1}). In subsurface flow CWs 73% removal of SMX was measured in an unplanted subsurface system (SSF), where influent concentration was 140 μg L^{-1}, while 87% was observed in a Phragmites SSF (influent concentration was 140 μg L^{-1}). Mean removal rates of 62-97% were obtained for three different treatment technologies in Mississippi, where SMX was detected in the highest median concentration of 1,640 ng L^{-1}. Treatment of wastewater effluent with ponds planted with Acorus followed by Typha plants, resulted in medium-range removal behaviour of SMX (approx. 80%), in the range of ppt. Wisconsin Fast Plants in a hydroponic study of Herklotz et al., (2010) were able to uptake SMX in the leaves, stem and roots, whereas, 138.26 ng g^{-1} of the compound detected in roots of cabbage.

Similarly, in the case of CIP, efficiency in the range 82-85% was measured in vertical subsurface flow CWs (Li et al., 2014b). A hydroponic experiment by Liu et al. (2013) revealed accumulation of CIP in tissues of *P. australis,* higher than that of oxytetracycline HCl and sulfamethazine.(Liu et al., 2013) High removal efficiency of CIP-HCl in a VSSF-CWs, at 82-85% is reported from Li et al. (2014), while 67% was reported for horizontal subsurface flow CWs. Contribution of wetland vegetation to fluoroquinolones removal has also been reported in previous studies (Hoang et al., 2013; Lillenberg et al., 2010).

Based on the above, contribution of *J. acutus* against antibiotics removal was remarkable, even in the presence of heavy metals up to medium concentrations. In this range, no visible toxicity symptoms was exhibited in the plants. Therefore, use of this species should be taken into consideration in case of urban and industrial wastewater treatment. In the case of pharmaceutical wastewater, where concentrations of antibiotics reach the highest values, *J. acutus* could be valuable, however, further research is needed in order to draw definite conclusions.

Heavy metals removal

Aqueous phase

Nutrient solution was analyzed every seven days to examine the fate of heavy metals in the aqueous phase. Removal efficiencies over the experimental period are shown in Fig. 22. The significant contribution of *J. acutus* at low concentrations of mixture of contaminants (E treatment) was indicated with the total removal of Cd, approximately 98% of Cr and Zn and 76% of Ni. Under medium levels (100x higher) of mixed contamination (F treatment) the concentrations of Cr, Ni, Zn and Cd were reduced by 58%, 86%, 94% and 100% respectively.

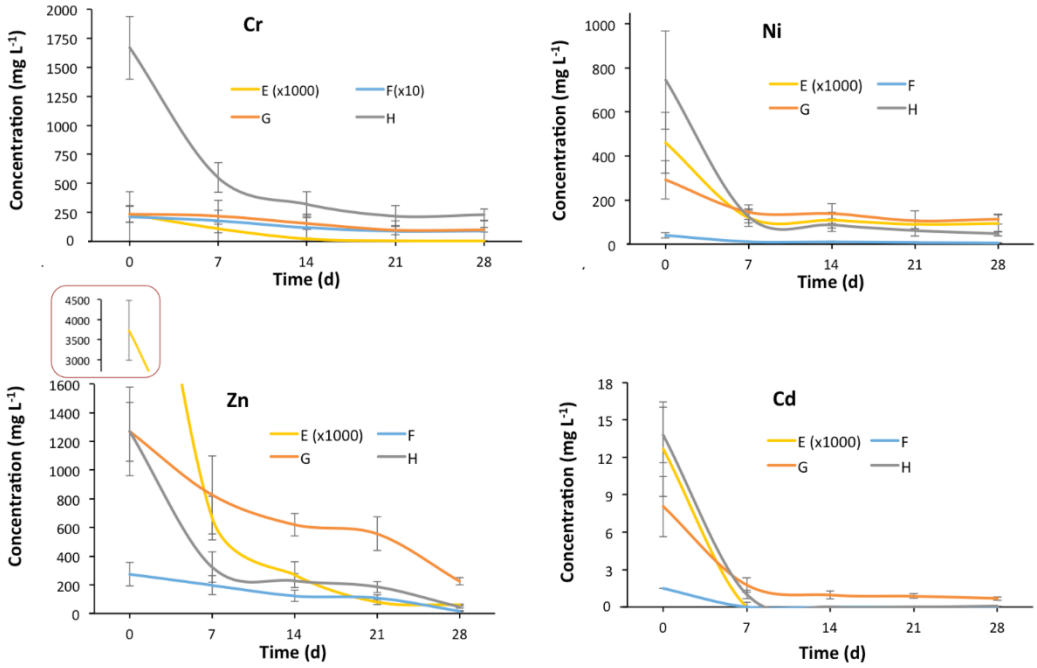


Figure 22. Heavy metals removal in the aqueous phase, over the experimental period. Values shown are means (n=7). Values of treatment E are multiplied by 1000 the real concentration.

Comparison of treatment G with H (only heavy metals), indicated the lowest efficiency of the first, especially in terms of Cr and Ni removal (58% and 61% respectively). Consequently, highest elimination in the aqueous phase was measured for Cd (>91%) regarding all concentrations tested. An overall reliable performance was observed for Zn (82%-98%), whereas, in the case of Ni, the lowest removal was 61% of the metal initially added (300 mg L^{-1} at G treatment). Fluctuation was observed in Cr removal that ranged from the lowest 58% (G treatment, initially spiked with 236 mg L^{-1}) to 98% (treatment E, spiked with 0.23 mg L^{-1}).

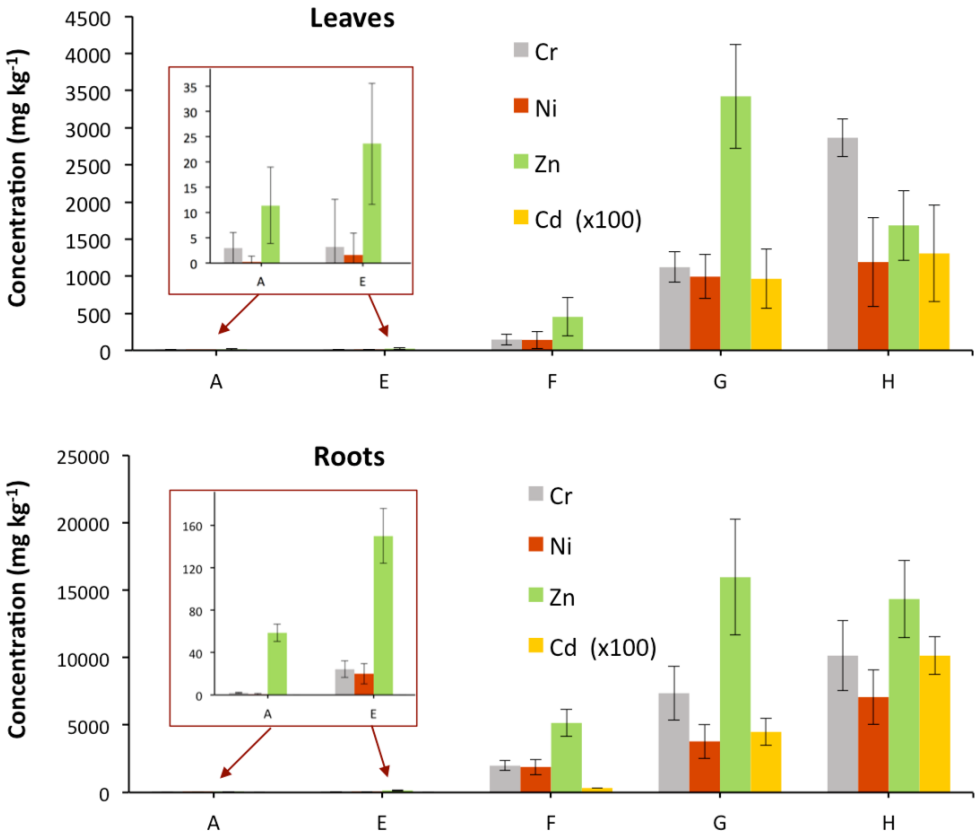


Figure 23. Heavy metals concentration in plant tissues (leaves and roots) at the end of the experiment (day 28). Values shown are means (n=7) with standard error bars.

Plant tissue

Roots and leaves of *J. acutus* were separately analyzed for metals uptake to determine the distribution of each metal within the plant (Fig. 23). Regarding Cr, 10148.0 mg kg^{-1}dw was the highest value detected (roots of treatment H), while 2865.9 mg kg^{-1} dw were found to be translocated to the leaves of the same treatment. TI showed the highest value in treatment H (41.7%) followed by G, E and F (29.5%, 18.4% and 17.6% respectively). Likewise, in a recent study of a constructed wetland experiment with *Juncus acutus* L. for Cr(VI)-contaminated groundwater treatment, the plant was found to be able to rhizofiltrate Cr(VI) with the majority found accumulated in its tissues.(Dimitroula et al., 2015) Moreover, in the same work, plant's endophytic community was found to able to biotransform Cr(VI) to Cr(III). Furthermore, *J. effusus* was also found suitable for constructed wetlands to treat chromium-containing wastewater.(Gruber et al., 2008)

In root tissues of treatment G, accumulation of Zn reached 15960.7 mg kg^{-1}, whereas 3424.0 mg kg^{-1} were translocated to the leaves. TI for Zn where not statistically significant different, in comparison to treatment F, which was found to have the lowest value. Similarly, in another

hydroponic study with Zn treatment of Mateos-Naranjo et al. (2014) *J. acutus* was found to be tolerant to high levels of Zn and this tolerance was associated by authors with its capacity to accumulate the metal in roots (values up to 2500 mg kg^{-1}) and largely avoid its translocation to shoots (values up to 500 mg kg^{-1}).

In the case of Ni, the maximum value of 7070.3 mg kg^{-1} was measured in the roots and 1190.3 mg kg^{-1} in the aboveground tissues of treatment H. On the other hand, in treatment G the highest TI (41.9%) was observed followed by H (29.9%) whereas, the lowest percentages (approx. 7%) were found for F and E.

Finally, regarding Cd, a concentration of 101.47 mg kg^{-1} was measured in root tissues and 13.07 mg kg^{-1} in the leaves in treatment H, showing the highest accumulation and translocation. In a river estuary study with Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn, *J. maritimus* was found to concentrate the metals around its roots but most of them appeared to accumulate in root tissues and not translocated in aerial plant parts and in comparison with all metals it showed a significant Cd bioaccumulation capacity.(Almeida et al., 2006) In a study of Najeeb at al., 2011 with Cd contaminated hydroponics and presence of chelators, *J. effusus* developed structural and morphological damage under Cd and EDTA treatment, while, citric acid had ameliorated Cd toxicity stress.

The results indicate that removal of the pollutant decreased with increasing initial concentrations of the metal in the nutrient solution (apart from Cd that showed the lowest values and no trend could be exported). Additionally, total pollutant's mass in the plant (roots and shoots) increased with increasing initial mass added in the treatment, despite the case of Zn, where mass translocated in the tissues was lessen when total mass of metals added in the solution increased (G and H treatments). These results suggest that even removal percentage is very low, as in the case of highly spiked treatments, the plant was able to translocate very high amounts of the metal. Future research should be conducted to provide further insight into where exactly the steady state conditions were reached and the boundaries that they set. Bioaccumulation coefficients decreased in the order of E > F > G > H, for all metals tested except Cd, showing for Cr the highest value of 47.4 ml g^{-1} at environmentally relevant concentrations.

In conclusion in this study, *J. acutus* has shown an ability to accumulate heavy metals in its tissues in considerable amounts. The concentrations in roots of plants were found to be extremely high as already shown in Fig. 23 and it is obvious that these measured concentrations indicate not only metal accumulation in the roots but also metal sorption on the root (Pivetz, 2001; Manousaki et al., 2009). Moreover, the plant seems able to even translocate them into its leaf tissues in significant amounts reaching in cases concentrations beyond the leaf

hyperaccumulation level; e.g. for Ni and Cr (1000 mg kg^{-1}) at the treatments with the highest metal concentrations (G and H). These high concentrations translocated to the shoots are most probably due to the effects of biosorption and other passive assimilation processes (Pivetz, 2001; Manousaki et al., 2009); however, it must be kept in mind that, in general, hydroponic studies merely give a general idea regarding metal tolerance of the plant and its metal uptake ability, rather than giving specific accumulation values (Pivetz, 2001; Manousaki et al., 2009).

Physiological responses of *J. acutus*

Most concern by international community has been elicited on plant's responses to heavy metals stress (Gruber et al., 2008; Nagajyoti et al., 2010; Yang and Chu, 2011; Gangwar and Singh, 2013) therefore, catalase (CAT) and guaiacol peroxidase (GPX) activity, proteins and chlorophyll content are widely used in phytoremediation studies handling heavy metals (Manousaki et al., 2008; Bhaduri and Fulekar, 2012). Although, an increased interest on how plants are affected by the presence of organic contaminants is currently noted. Among them, petroleum hydrocarbons and emerging organic contaminants, including pharmaceuticals and pesticides take hold a great portion, as shown from latest studies (Carvalho et al., 2014; Prasad et al., 2014; Gutiérrez-Ginés et al., 2014; Wang et al., 2011). Furthermore, BPA has attracted the concern in this field, where, as seen for example in a study of Ferrara et al. (2006), it could influence morphological and physiological status of seedling of various crops, when exposed to 10 and 50 mg L^{-1}.

Table 13. Average dry weight of roots and leaves per plant, as measured at the end of the experimental period. Mean values are reported (n=7).

Treatment	Leaves dry weight (g)	Roots dry weight (g)
Control	1.45	0.76
B	1.23	1.11
C	1.04	0.82
D	2.93	1.16
E	2.85	1.67
F	2.29	0.79
G	3.21	1.17
H	2.50	0.99

It is reported that chlorophyll content decreased in the presence of organic contaminants in some species (Xiao et al., 2010), while BPA at high levels (e.g., >7.0 mg L^{-1}) led to a decrease in chlorophyll pigment content in soybean seedlings (Qiu et al., 2013). Considering antibiotics, chlorophyll content is reported as a parameter for evaluating the toxicity caused by pharmaceuticals (Li et al., 2014b). Wang et al. (2011), used chlorophyll content, catalase and

peroxidase activity to evaluate the phytoremediation potential of reed against diesel contamination. It is also mentioned by Liu et al., that changes in antioxidant enzyme activity can be observed due to organic contaminants. Additionally, antioxidant enzymes CAT and GPX are reported as stress indicators from pharmaceutical compounds from Li et al. (2014b), while another paper concluded the relation of free radical accumulation with organic contaminants, as a result of antibiotics contamination (Liu et al., 2011). Liu et al. (2013) in a hydroponic experiment with *Phragmites australis* report increase of peroxidase and decrease of superoxide dismutase and catalase activity, as antibiotics dosages increased.

Table 14. Concentration of heavy metals in the tissues, total mass in the plant, translocation index, percentage of total initial pollutant (in the nutrient solution) and bioaccumulation coefficient for all treatments. Mean values are reported (n=7).

Treatment	Concentration in the tissue ($\mu g\ g^{-1}$)		Pollutant's mass in the plant (μg)	% removed of total initial pollutant	Bioaccumulation Coefficient ($ml\ g^{-1}$)
	leaf	root	$C_p m_p$	$C_p * m_p / (C_{nutr} * V_{nut})$	$BC = C_p / C_{nutr}$
			Cr		
E	3.23	24.35	50	61.28	47.40
F	145.92	1988.68	1902	25.54	29.00
G	1121.41	7363.06	12213	14.77	11.79
H	2865.88	10147.90	17178	2.94	2.95
			Ni		
E	1.61	20.03	38	23.70	18.33
F	141.68	1892.91	1817	13.26	15.06
G	993.94	3788.95	7625	7.49	5.98
H	1190.30	7070.35	9952	3.82	3.83
			Zn		
E	23.62	149.90	318	24.38	18.86
F	451.61	5156.81	5101	5.30	6.02
G	3424.01	15960.69	29666	6.67	5.33
H	1684.45	14344.31	18365	4.14	4.16
			Cd		
E	<D.L.	<D.L.	0	-	-
F	<D.L.	3.35	3	0.51	0.58
G	9.65	44.94	84	2.97	2.37
H	13.07	101.47	133	2.75	2.76

In this study, measurements of chlorophyll content, biomass and daily water consumption of *J. acutus* were conducted, whereas, production of CAT, GPX and protein content were determined, to evaluate the physiological state and indicate oxidative stress in plants. Furthermore, no visible signs of toxicity stress such as chlorosis or roots color changes were visually noted during the experiment.

Regarding total chlorophyll, statistically significant differences comparing with control, were found in F, G and H treatments, i.e. these containing a mixture of metals above medium level (Fig. 24). Comparison between spiked treatments revealed that chlorophyll content dropped significantly in three cases; firstly, when antibiotics concentration increased from medium to

high (C-D), secondly, when metals concentration increased from environmentally relevant to medium concentrations (E-F), and finally with the addition of metals keeping constant antibiotics concentration, in both medium and high values of the contaminants (C-F & D-G). In accordance with chlorophyll, statistical analysis indicated significantly lower average water consumption in G treatment (58.5 mL day^{-1}) compared with unspiked plants (122.5 mL day^{-1}). These results showed that under environmentally relevant concentrations of both organic and inorganic contaminants, plants were not negatively affected.

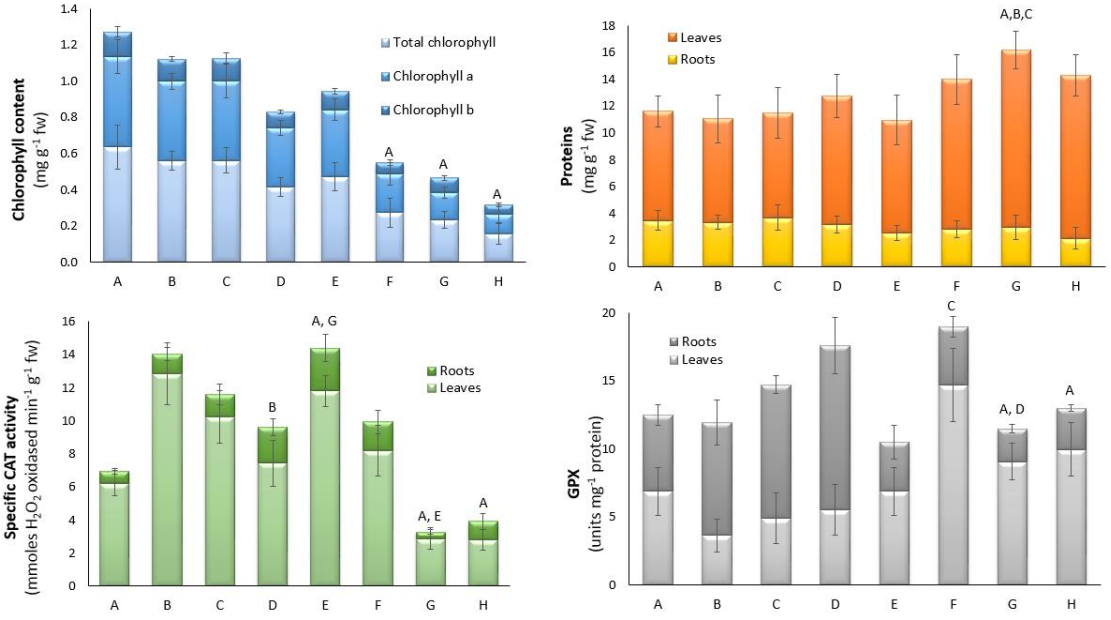


Figure 24. Evaluation of the physiological state of plants expressed in terms of chlorophyll content, protein content, catalase activity and specific guaiacol peroxidase activity, measured at day 28. Values shown are means (n=7) with standard error bars. Letters indicate significant (P <0.05) differences.

Physiological changes in plants were also evaluated by measuring biomass, expressed as roots and leaves dry weight after harvesting. Neither root and leaves nor total dry biomass resulted in significant differences among the treatments (data not shown). By examining the protein content, difference measured between the leaves of G treatment, exposed in the highest concentration of co-contamination and control treatment. Besides, protein content in the leaves of G treatment differed from those of treatments B and C. In particular, 8.1 mg g^{-1} of fresh leaves was found in controls, 7.7 mg g^{-1} in B treatment, 7.8 mg g^{-1} in C and 13.3 mg g^{-1} in G treatment. Proteins in the roots did not possess significantly different average values among the treatments.

Concomitantly, catalase activity in the leaves of *J. acutus* is shown to be similarly affected in both cases, under only antibiotics and mixed contamination treatments (Fig. 24). As antibiotics

or mixture of contaminants concentration in the nutrient solution increased, catalase activity significantly declined from 12.8 mmol min^{-1} g^{-1} (Standard error (SE): 1.9) to 7.4 mmol min^{-1} g^{-1} (SE: 1.4) (B, D treatments) for the first case and from 11.8 mmol min^{-1} g^{-1} (SE: 0.9) to 2.8 mmol min^{-1} g^{-1} (SE: 0.5) in the case of mix contamination (E, G treatments). Additionally, significant impact with respect to non-treated plants was measured in treatments G and H, i.e., highly spiked treatments with mixture of contaminants (G) or only with heavy metals (H: 2.8 mmol min^{-1} g^{-1} (SE: 0.6)).

Catalase production in the roots did not follow the status in the above ground tissues. Significantly elevated activity for catalase, was observed only for the treatment corresponding to environmentally relevant concentrations of contaminants (treatment E: 2.6 mmol min^{-1} g^{-1} (SE: 0.8), compared to the control. Analysis among the treatments showed a significant change in catalase production in the above mentioned treatment E and G (0.4 mmol min^{-1} g^{-1}, SE: 0.09).

GPX in the below ground biomass showed a decline in all mixed contaminant treatments (Fig. 24), although differences were found only between four comparable pairs of treatments: the control (5.6 U mg^{-1}, SE: 0.8) and the high concentration mixed treatment (G: 2.4 U mg^{-1}, SE: 0.4), the control and the high concentration heavy metals treatment (H: 3.0 U mg^{-1}, SE: 0.3), the two medium concentrated treatments with antibiotics (C: 9.8 U mg^{-1}, SE: 0.7) and mixed contamination (F: 4.3 U mg^{-1}, SE: 0.7), and finally between the two treatments with the highest addition of antibiotics (D: 12.1 U mg^{-1}, SE: 0.3) and mixed contamination (G). Regarding GPX in the leaves, no significant difference was measured when control was compared with spiked treatments.

Concluding remarks

In this study, *J. acutus* was exhibiting a significant contribution on the removal of CIP and SMX. When nutrient was spiked only with antibiotics, at the highest concentration tested of 10 mg L^{-1} for CIP and 50 mg L^{-1} for SMX, 92% and 61% removal was observed respectively, after 28 days. In the mixture with heavy metals and BPA, reaching extremely high concentrations, removal was more than 71% for CIP and 87% for SMX. Resultant removal capacity of BPA was great (>96%), even when plants were exposed to 50 mg L^{-1} of the compound and concentration of heavy metals reached the highest levels. Additionally, *J. acutus* has shown an ability to accumulate heavy metals in both root and leaf tissues, in considerable amounts, reaching, in cases, concentrations beyond the leaf hyperaccumulation level. The plant was able to accumulate high levels of heavy metals, even when the percentage of metal's removal was low, as measured in the highly contaminated treatments. Tolerance of the plant was also revealed, after its very sufficient response to organic and inorganic stress.

Remarkable efficiency of *J. acutus* was revealed, regarding all the contaminants tested in environmentally relevant concentrations. Hence, contribution in environmental problem through CW systems, seems to be substantial. In higher concentrations of antibiotics and mixture of organic and inorganic contaminants, thus wastewater derived from pharmaceutical companies or industrial facilities, *J. acutus* may also perform a competitive role. Thus, it is demonstrated as a high promising candidate for phytoremediation applications, in CW systems treating wastewater of a wide range of sources.

5.2. Shallow Aquifer Rhizodegradation pilot

Runs R (August 2012) and Q (July 2013) allow a comparison under almost the same conditions, but with a year's difference. In both runs 160 mg of BPA added in the external vessel at day zero, corresponding to an initial concentration of 2.67 mg L^{-1}. As seen in Fig. 25, BPA concentration starts rising after 4 days of no detection, until day 9 when it takes the highest value of 5.82 μg L^{-1} and then a decline in BPA concentration is observed until the 13th day and after then it is no longer detected. In the case of the latter experiment, BPA concentration removal versus time appears to have a different profile, since it shows a very early peak (day 2) pertaining to a much higher BPA concentration. This obvious offset in the time of peaks appearance and magnitude, was observed for all treatments followed Q run, something that was attributed to a modification of the influent distributor (bottom pipe network was replaced from a single point influent) and also to the channels that were created between the walls of the pilot and the soil and have caused earlier overflow of the contaminant from the tank to the surface, prevent passing gradually through the soil compartment and consequently could cause a reduction in the concentration.

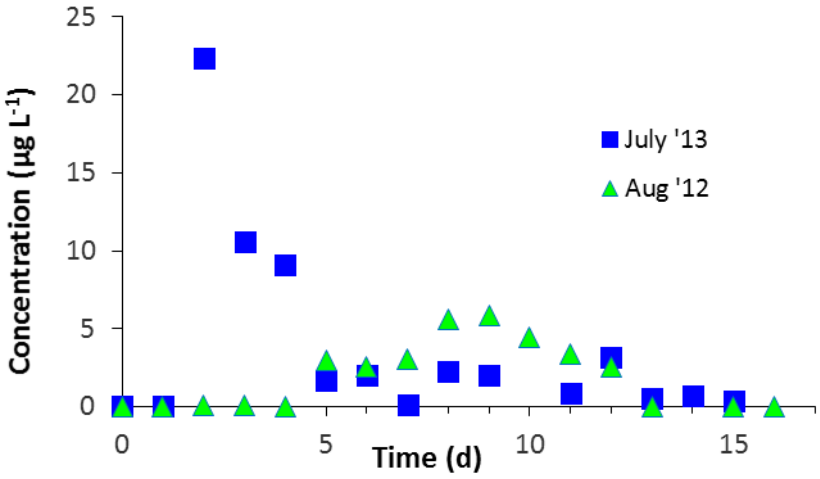


Figure 25. Comparison of the concentrations of BPA during different runs.

Except for this phenomenon in the first days of both experimental runs, BPA concentration was not detected after 15 days. In the later experiment, we observe that BPA concentration remains at approximately 2-2.5 μg L^{-1} between days 5 and 11, when it starts to decrease. However, there is little fluctuation as seen in the graph (days 7, 11 and 12), notwithstanding, it is considered within acceptable limits, since dealing with concentrations of μg L^{-1} in soil substrate. In Fig. 25, it is clearly shown that overflow concentration of BPA decrease faster in the later experiment. This could be attributed to the growth of plants, thus higher root density and corresponding associated microbial growth (Petoussi et al., 2014).

BPA concentration dynamics in the aqueous phase of run R, was computed after conceptually divining of the tank volume vertically, into four different compartments (Fig. 26). The first layer at the bottom of the pilot is considered to exclusively consist of gravel, whereas BPA concentration of the upper one is equal with the measured concentration in the effluent of the system (the sampling point before overflowed water enters into the external vessel (Fig. 8). Description of the experimental procedure is given in the article written by Petoussi et al. (2014). It is easily noticeable from Fig. 26 that in the case of the gravel compartment (bottom part) BPA concentration reaches its peak of approximately 12 μg L^{-1} more rapidly than in the other compartments. This could be explained by the following reasons: firstly, the highest hydraulic conductivity in the gravel layer, secondly the lower biodegradation in this compartment due to the absence of roots and the associated microorganisms and finally because of less sorption in gravel than in soil particles of high organic matter.

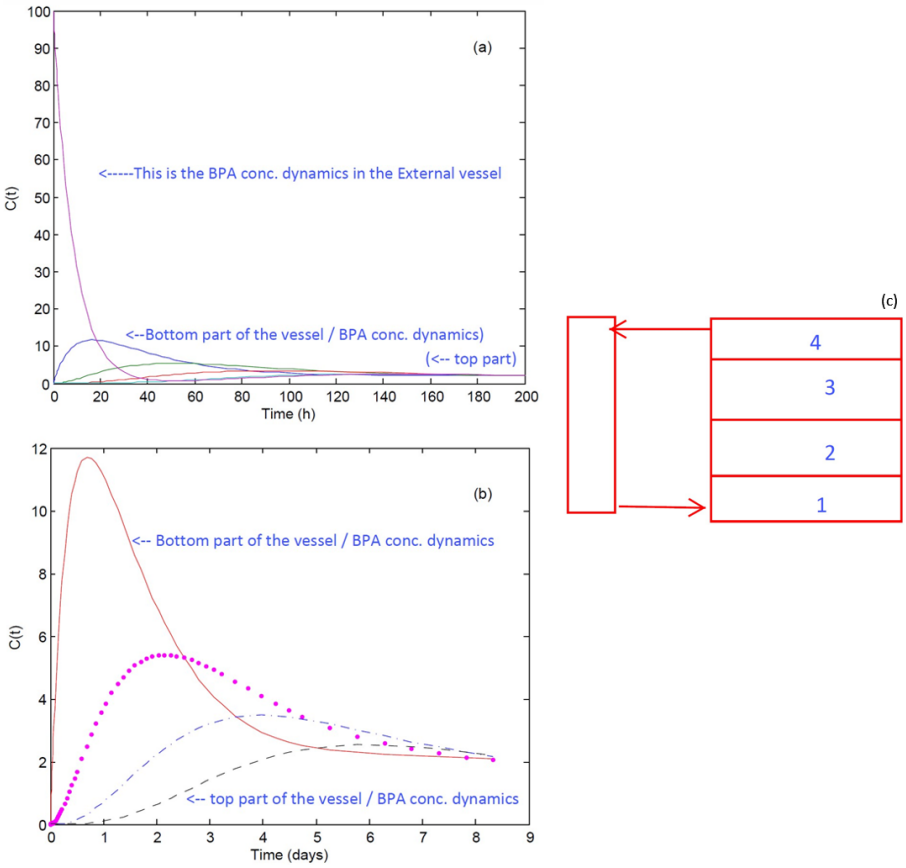


Figure 26. BPA concentration until day 8, in the aqueous phase of the four compartments of the soil layer of rhizodegradation pilot and the external vessel (a) and BPA dynamics in the pilot, in larger scale (b). Layer distribution of the pilot unit (c).

Additionally, it is observable that maximum BPA concentration takes lower values as the referring compartment is higher. This is ascribed to biodegradation and sorption of the compound as water passes through the layers. Moderate high hydrophobicity of BPA (log K_{ow}

= 3.32), supports sorption onto the organic matter that retained in the substrate (Papaevangelou et al., 2016; Ávila et al., 2013) thus, it is expected a lower removal rate in the bottom layer. What is more, after BPA takes the maximum concentration in this layer, removal rate from the aqueous phase is almost constant until it starts to diminish, pertaining low levels of BPA concentration.

With reference to robustness, a system can be characterized as "robust" if it is able to recover easily after an oscillation of its nominal conditions. For this reason, a robust system is characterized for overcoming whenever a disturbance takes place within a brief time period. In these conditions, the systems performance is not only affected by the deviations of the system input, but depends also on the duration of the input variations. Even though a range of factors may complicate the in situ (bio)remediation process, adaptability and resilience may play a significant role as for instance the ability of microbial communities to activate mechanisms of survival in order to front intense fluctuations in their environment.

Therefore, in order to estimate the robustness of the rhizodegradation system, experimental runs 2Q and HC where high BPA concentration and volumetric inflow rate applied, have been performed to be compared with the nominal case. Rhizodegradation system faced a disturbance after the influent volumetric flow rate switched to double, even keeping constant spiked BPA mass. With reference to Fig. 24, doubling of the influent flow rate, yielded to an offset in time of BPA maximum concentration detection (day 1) as expected to be, since the potential of BPA transfer from the aqueous phase to the soil and the effect of biodegradation, were lower.

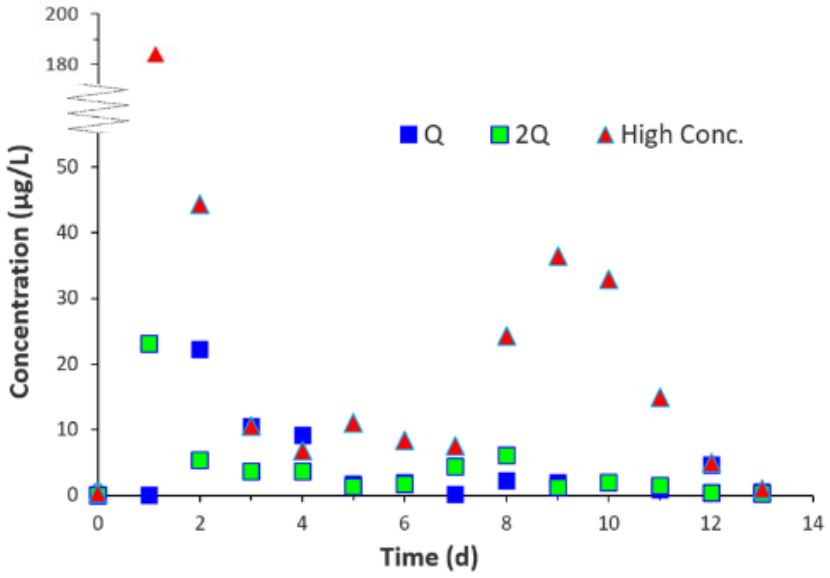


Figure 27. Comparative evaluation of BPA concentration under nominal conditions (Q), double flow rate (2Q) and increased initial BPA mass addition (HC).

When testing the scenario of the excessive BPA mass spiked into the system, together with applying high volumetric flow rate (run HC), the system responded positively. More specifically, addition of 5 g of BPA induced a sharp rise in BPA peak after 24 h from the day of spiking, corresponded to 184 μg L^{-1} in the effluent. A rapid decrease in concentration was followed by a second peak at day 9. Indeed hydrophobic substances are known to be bound to the organic matter. Sorbed organic compounds are less bioavailable and accessible to microorganisms, thus more recalcitrant to biodegradation (Paranychianakis et al., 2006). Additionally, soils from sites exposed to organic pollutants for a long period of time, has been reported to demonstrate different biodegradation rates than freshly spiked soils (Reichenauer and Germida, 2008). These facts suggest that the second peak at day 9 could possibly indicate desorption of BPA from soil particles. Nonetheless, overflow BPA concentration dropped below the detection limit at day 14.

In brief, the system showed for all cases to be very robust as it has the capability to assimilate the BPA mass only in the first few days. It should be reminded that repeatability of the process performance was confirmed by the repetition of runs R and 2Q (data not showed) that resulted to no significant difference in concentration's removal profile.

Concluding remarks

The application of rhizodegradation has been proven to be not only a very challenging, but also a low cost and efficient method for the removal of BPA from contaminated groundwater, despite some limitations (i.e., the contaminated groundwater must be reachable by the roots of the selected plants, all contaminated groundwater must pass through the root zone of the planted trees, suitable geological & flow characteristics of the soil).

In this experiment, halophytes developed no visible toxicity symptoms as well as no growth inhibition in the presence of BPA, even at concentrations above measured field values. So, it is supposed that endophytic bacteria, available in both root & leaves, could enhance the degradation of BPA and as a result this leads to the effective detoxification.

Overall, the shallow aquifer rhizodegradation system has been shown to be reliable and robust within reasonable input oscillations of the BPA concentration and inlet volumetric flow rate. Furthermore, flow bypass seems to protect the system from deterioration at extreme oscillations, because they decrease possible negative effects for the system which being faced with an extremely high concentration. This result means that in the field, the effect of BPA contaminated plume will be parceled to the root system of more than one plants. Also, a bypass channel seems to function as a buffer tank for the system.

5.3. Small scale HSF-CW for secondary treated municipal wastewater

5.4.1. Initial evaluation concerning BPA removal

In this experiment, a first evaluation of the system to remove BPA in operation with HRT set at 2 days and constant BPA loading, was attempted. The HSF-CW was able to decrease BPA influent concentrations of 3.2 and 4.2 μg L^{-1} at 77 and 75% for L1 and L2 treatments respectively (Fig. 28). BPA concentration at the influent during the whole experimental period, demonstrated a variation from 1.36 to 8.16 μg L^{-1}. Effluent BPA concentration was lower than the influent during all the experimental procedure for both treatments.

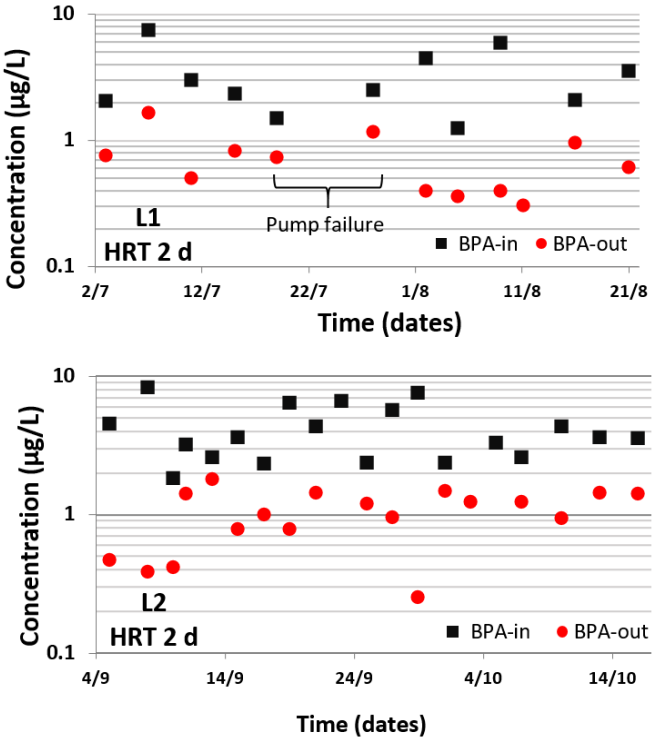


Figure 28. Bisphenol A concentrations in the influent and the effluent of the planted HSF-CW over time, in logarithmic scale. Time period: summer and autumn, 2015.

Removal performance of the wetland during summer was slightly better, though, a non–parametric, Mann-Whitney test among the two treatments, did not result in statistical significant difference ($p < 0.05$). During L1 treatment, operation of the system was interrupted, due to BPA feed pump failure. Overall, these results are in accordance with those of similar studies dealing with EDCs removal by CWs. However, since there is a large number of parameters involved, a wide variety of results is found in the bibliography. For instance, Tore et al. (2012) reported a BPA removal of 0.05-0.3 μg L-1, in the range of 80–100%, by a full scale HSF-CWs. On the

other hand, Toro-Velez et al. (2016) using a small-scale HSF-CW system planted with Phragmites australis, demonstrated similar removal efficiencies in the level of 70%. Possible mechanisms responsible for the removal of BPA in the wetland are discussed in the following section.

5.4.2. Investigation of the impact of vegetation on organic contaminants removal

In this part of the experimental procedure a comparison between the control unit (absence of vegetation) and the vegetated one, was firstly attempted in order to evaluate the contribution of plants on the contaminants' removal. Table 15, illustrates the time period, meteorological data as they were monitored in a three hour step, daily total estimated evapotranspiration, measured influent and estimated effluent flow rates.

Table 15. Information on each experimental run at the HSF-CW: time period, meteorological conditions, evapotranspiration (ET) and the calculated input and output flow rates, the latter are corrected in respect to evapotranspiration.

Experimental run	HRT	Month	Air temperature (^{o}C) / Relative humidity (%)	ET (L d^{-1})	Q_{in} (L h^{-1})	Q_{out}^{adj} (L h^{-1})
Control	1	February	14.4 / 67.6	-	2.37	2.37
T	1	March	16.2 / 65.9	20.0	6.54	5.71
HR1	1	April	21.9 / 57.5	25.0	6.54	5.50
HR2	2	June	29.7 / 53.1	32.5	3.27	1.92
PR	2	July	29.8 / 54.2	30.0	3.27	2.02
OB	2	December	18.3 / 66.1	25.0	3.27	2.23

BPA removal

Planted and non-planted mesocosms were operated under the same nominal influent BPA concentration (100 μg L^{-1}) and HRT (one day), as well as approximately equal environmental conditions (air temperature and humidity). Additionally, both wetlands were covered to prevent rainwater input. Average temperature and humidity during treatments differed for 1.8 ^{o}C and 1.7%, respectively. Results are summarized in Fig. 29, in which concentration of BPA for both wetlands during the winter period is plotted as a function of time, for both cases. The positive effect of vegetation is undoubtedly apparent. Oscillation of the influent concentration could be attributed to the pulsed pump operation for both wastewater and spiked contaminants influent, as well as to BPA naturally occurred in wastewater.

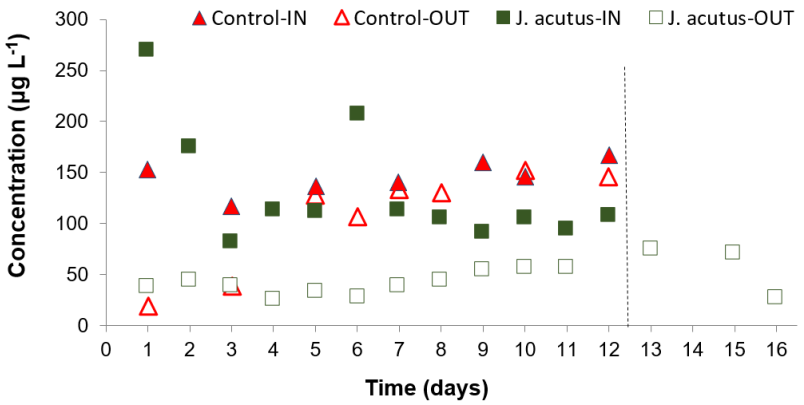


Figure 29. Bisphenol A concentrations in the influent and the effluent of the planted and non-planted (control) horizontal subsurface flow wetlands, during winter. Green squares represent BPA concentration, corrected in respect to evapotranspiration. Dotted line indicates interruption of spiking.

Statistical analysis of the results was carried out as follows: significance of the concentration removal for each experimental run (C_{in} and C_{out}^{adg}) was estimated after checking normality of the data using Shapiro–Wilk test. Variables of both experimental runs, control (non-planted) and T (planted with *Juncus*), satisfied the normality assumption (with 1000 iterations of bootstrap analysis, $p < 0.05$) therefore, paired sample t-test was performed to test the significance of each unit. Results show that concentration at the effluent was significantly lower than at the influent only for the vegetated unit (p=0.028, Table 16). The above results outlined the contribution of plants and their associated microorganisms in the removal of the endocrine disruptor. Rhizosphere, the reactive zone of CWs, is the area where the interactions of plants, microorganisms, xenobiotics and the substrate take place (Stottmeister et al., 2003). Previous studies on subsurface flow of CWs have shown that oxygen plays important role in EDCs' removal, giving an advantage in vertical flow systems. However, both aerobic and anaerobic processes that take place near roots in HSF environments could lead to multiple degradation pathways (Papaevangelou et al., 2016). BPA removal did occurred also in the control pilot wetland, but it was measured to be significantly lower due to fewer degradation pathways. Furthermore, sorption onto the biofilm developed on the gravel media, appeared to be the prevailing abiotic mechanism for the contaminants removal (Dordio and Carvalho, 2013). However, it should be taken into consideration that short HRT applied into the system, does not favor sorption onto the biofilm produced in the gravel and root surface.

Antibiotics removal

With reference to Table 16 and Fig. 30, it can be seen that efficiency of the wetlands in terms of antibiotics removal, is slightly variable between ciprofloxacin (CIP) and sulfamethoxazole (SMX). Considerably high removal of CIP was observed in the vegetated unit, although there is also a noticeable decrease in the absence of plants. These results are consistent with our primary hydroponic experiment, in which natural attenuation of CIP in the control treatment varied from 59 to 54%. Non-parametric Mann-Whitney U test resulted in significant difference in CIP concentration removal between the two mesocosms ($p = 0.004$). Mass removal rate was calculated at 7.4 mg h^{-1} for the vegetated and 1.1 mg h^{-1} for the control wetland respectively, a difference that is partially attributed to the higher flowrate of treated wastewater in the vegetated wetland, as stated previously. Biodegradation in the substrate, as well as sorption onto the biofilm of gravel medium are proposed as possible mechanisms of the removal, in accordance with previous studies (Avila et al., 2013). However, Liao et al. (2016) given the fact that ciprofloxacin dissipation by microorganisms is yet to be fully understood, identified microbiota that could utilize CIP as their sole carbon and nitrogen source and proposed four biodegradation pathways of the compound. Overall, this review revealed that fluoroquinolone antibiotics have complex behavior during wastewater treatment and exhibit incomplete removal.

The sulfonamide antibiotic displayed a different behavior in the CWs. Fig. 30 illustrates variations of concentrations and insufficient removal of SMX, even in the vegetated wetland. This is reflected by the term m_p that is below 20%. In the case of control treatment no removal was noted, with some individual values of negative removals. This phenomenon has been also addressed in previous studies and it will be further discussed in a following section of this unit. Statistical analysis of the results, using Mann-Whitney U test ($p < 0.05$, Monte Carlo bootstrapping based on 10000 sampled tables) indicated that there was no significant difference between the two wetlands regarding SMX concentration removal ($p = 0.074$).

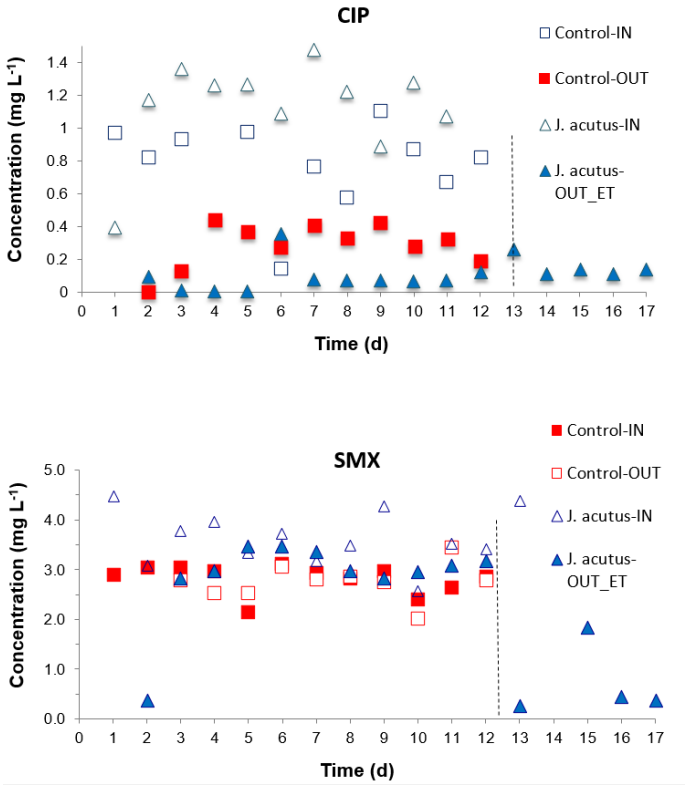


Figure 30. Comparative presentation of antibiotics concentration versus time, for the planted and non-planted mesocosms. ET indicates that corresponded values have been corrected in respect to evapotranspiration. Dotted lines indicate the interruption of spiking.

Table 16. Comparative presentation of the efficiency of the two constructed wetlands: the control (in the absence of plants) and the *J. acutus* planted wetland. Retention time (HRT) in both experiments is one day. C_{in} and C_{out}^{ET} are the mean measured concentrations at the influent and the effluent of the wetlands (12<n<13). ET indicates that corresponded values have been corrected in respect to evapotranspiration. m_p is the removed mass of the contaminant over the mass entering the system. In the parenthesis is p-value, with significance level set at p < 0.05.

Organic compound	Treatment	Measured influent concentr. BPA (μg L^{-1}) CIP, SMX (mg L^{-1})	Measured effluent concentr. BPA (μg L^{-1}) CIP, SMX (mg L^{-1})	Concentration removal (%)	Concentration removal ET adjusted (%)	Significant difference (C_{in}-C_{out}^{ET})	Rate of mass removal ET adjusted BPA (μg h^{-1}) CIP, SMX (mg L^{-1})	m_p (%)
BPA	Control	146.0	106.7	26.8	26.8	No (0.115)	93.0	26.9
	T	131.0	41.7	68.2	76.2	Yes (0.028)	618.5	73.2
CIP	Control	0.79	0.32	59.8	59.8	Yes (0.006)	1.12	60.0
	T	1.21	0.10	91.9	93.9	Yes (0.003)	7.38	93.1
SMX	Control	2.8	2.77	1.1	1.1	No (0.466)	0.07	1.2
	T	3.6	3.52	3.0	27.2	No (0.180)	3.64	18.5

The role of vegetation in CWs

The role of vegetation has been addressed in several studies on EOCs removal from wastewater with CWs. In a literature review concerning the efficiency of biologically based wastewater treatment systems for the removal of EOCs, the positive contribution of macrophytes in SSF-CWs is outlined (Garcia-Rodríguez et al., 2014). The essence of oxygen release from plant roots is highlighted as a vital process for the enhancement of biodegradation in the rhizosphere, due to the created aerobic pathways. The amount of oxygen release could reach sometimes the 90% of the total oxygen in the substrate (Allen et al., 2002). It is also known that plants are able to oxidize phytotoxic reduced compounds in the rhizosphere (Fe^{2+},Mn^{2+},S^{2-}), by transporting oxygen into their roots (Allen et al., 2002). Besides, helophytes excrete root exudates to the rhizosphere (sugars, amino acids, vitamins and other organic compounds) that stimulate microbial growth, a process known as rhizodeposition (Stottmeister et al., 2003). Furthermore, root exudates influence degradation of organic contaminants, through bioavailability of the latter, which is promoted because of the provided growth substrate and the increased population and activity of microorganisms (Paranychianakis et al., 2006). Nevertheless, plant uptake is considered to be a mechanism of minor importance for the removal of organics by CWs in comparison with biodegradation, especially for the moderate hydrophobic BPA (Stottmeister et al., 2003). The above theory is in a good agreement with other researches. As an example, the analysis of seven small-scale CWs dealt with primary treated urban wastewater contaminated with PPCPs, revealed no significant correlations ($p < 0.05$) between PPCPs removal efficiencies and the uptaken concentrations, demonstrating in this way that plant uptake/adsorption was insignificant (Hijosa-Valsero et al., 2016).

Possible removal mechanisms of EOCs in the HSF – CW system

Various mechanisms could be incorporated to the depuration of pharmaceuticals in a CW, including physical (such as retention, volatilization and adsorption onto the substrate's biofilm and roots), chemical (break down of the contaminants) and biological (plant assisted rhizoremediation, plant uptake, oxygen and exudates release into the rhizosphere) (Carvalho et al., 2013). Hydrolysis is not expected to contribute to the attenuation under environmental conditions, since the endocrine disruptor does not contain functional groups susceptible to hydrolysis (Fent et al., 2003). In a research article focusing on the removal of EOCs by HSF-CWs, biodegradation and sorption onto the substrate, are presented as the main removal mechanisms of BPA from contaminated wastewater, accenting the former as the predominant among them (Ávila et al., 2010). In this regard, it is consider by several authors that optimum conditions for the removal are not obtained in the HSF- CW, since, although biodegradation is demonstrated the major removal mechanism, especially under aerobic conditions, these conditions are not prevailed in a bed of a horizontal system (Ávila et al., 2010)(Al-Rifai et al.,

2007). Furthermore, in the extensive review article of Garcia-Rodríguez et al. (2014), adsorption onto solid particles is noted as the key removal mechanism of BPA in HSF-CWs planted with macrophytes, due to its relatively high hydrophobicity (log K_{ow} > 3.5). In accordance with Avila et al. (2014), Papaevangelou et al. (2016) points out the vital role of retention onto particulate matter in the substrate of HSF-CWs (filtration, sedimentation and adsorption), with emphasis on the presence of oxygen and the coexistence of aerobic and anaerobic degradation pathways, due to the presence of both aerobic and anaerobic conditions in the system. Hijosa-Valsero et al. (2016) recently confirmed biodegradation pathways of several PPCPs during treatment with CWs, after identifying their transformation products. They concluded that biodegradation of pharmaceuticals is suggested to be the main mechanism for most of the systems tested in this study. A different aspect, the density of plantation, is also addressed for the removal, as it induces lower hydraulic conductivity and therefore, reduced transport of the contaminants (Schulz et al., 2003). Based on our experience working with *J. acutus*, especially in the present CW experiment, we believe that rapid growth rate, remarkable rhizomateus growth rate and root density, as well as overall robustness of this species, more than satisfy the above requirements (Syranidou et al., 2016).

Taking a closer look at CIP removal, in an extensive research review on fluoroquiolone (FQ) antibiotics, is documented that the role of biodegradation in the removal of this group of pharmaceuticals remains to be resolved (Van Doorslaer et al., 2014). Though, concerning WWTPs, several authors agree that predominant removal mechanisms of FQs is sorption to sludge, rather than biodegradation. Hydroponic experiments described by Liu et al. (2013), and the presented thesis both confirmed the the contribution of plants to the removal of CIP. Hydrolytic action and, to a much lesser extend, photodegradation were desrcibed as the two key mechanisms of CIP concentration removal. As a consequence, it is stated that free water systems (FWS) tackles with pharmaceutical pollution more efficiently compared to subsurface system, where contaminants are not exposed to direct sunlight (Zhang et al., 2014a). The enhanced reduction of CIP in the non-planted CW seems to be due to the substrate: gravel provides good hydraulic conductivity and is more resistant to clogging (Upadhyay et al., 2016; Wu et al., 2015). Gravel bed of the non – planted HSF, seems to facilitate biofilm production, microbial growth and redox conditions that contribute to the removal, whilst, most manufactured organic compounds are considered to be enhanced under aerobic conditions (Dordio and Carvalho, 2013).

With respect to SMX removal mechanisms, high pKa values of sulfonamides result to ehanced electrostatic interaction and therefore, efficient adsorption. In this regard, microcosm experiments have revealed the important role of microorganisms to SMX degration in CWs through the presence of plants (*P. australis*). Actually, authors suggest that biodegradation by

microorganisms present in plant material, as far as adsorption to soil particles and plants, are the main mechanisms mediated for the removal (Choi et al., 2016). In a CW experiment in Korea, initially developed for livestock wastewater treatment, 49% removal of the 10-11.6 μg L^{-1} at the entering to the system, was reported (Choi et al., 2016). Nonetheless, much lower removal is reported in several other studies, as it is furtherly discussed in the following section.

5.4.3. Efficiency of the constructed wetland under different operating conditions

Impact of HRT on organic contaminants removal

Removal of BPA and possible mechanisms involved

Figure 32 presents a scatter diagram of BPA concentration at the influent and effluent of the wetland versus time. It is important to mention that HRT values are not adjusted in respect to evapotranspiration, but refer to the influent flow rate of the wetland. Mean BPA concentration removal was measured at 48% at HRT 1 day and reached 92% when HRT was doubled (influent measured concentrations were 4.1 and 3.1 μg L^{-1}, respectively) (Table 18). As expected, mass removal rate at HRT 2 days was found less than in half HRT. Specifically, it was estimated at 12.8 μg h^{-1} in low and 9.4 μg h^{-1} in high HRT. In high HRT the contaminant remains longer in the rhizosphere which is the active zone of the wetland, giving in this way more time to biotic removal processes to escalate (biodegradation and phytoremediation), whereas, adsorption on the organic matter is also enhanced. On the contrary, at high HRT, wastewater flows rapidly through the wetland, reducing the contact time with the root zone and plant associated microorganisms (Zhang et al., 2014a). Though, mass removal rate is highly dependent on the influent flow rate and concentration, as derived from Eq. 20. The first was by definition higher in HRT 2 d and influent concentration was measured slightly higher in the same experimental run, hence, affecting the resultant removal rate. Considerable variation on the influent concentration has been also reported in studies on EDCs treatment from municipal wastewater with CWs, at concentrations up to an order of magnitude greater than the spiked in case of BPA (Toro-Velez et al., 2016; Al-Rifai et al., 2007; Lee et al., 2009). The superior treatment performance of the wetland when HRT was 2 d is emphasized by m_p ratio that estimated 92%, compared to 48% at low HRT and it was statistically corroborated using independent t-test (with 1000 iterations of bootstrap analysis, $p < 0.05$) that yielded to the significant different removal among the two operating conditions.

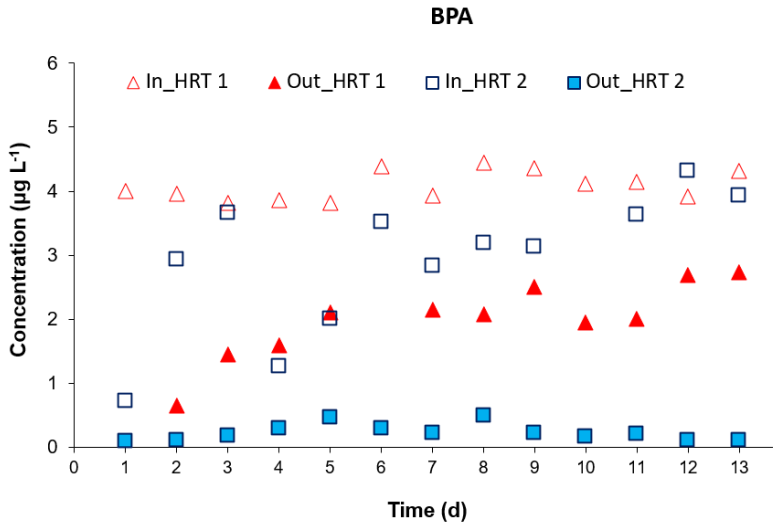


Figure 31. Comparison of the influent and effluent BPA concentration versus time, with respect to different HRTs.

Hydraulic residence time, is known to have substantial role in removal efficiency of organic contaminants (Stottmeister et al., 2003). Like in our case, Toro-Vélez et al. (2016) used three HSF-CW (9 x 3 x 0.6 m) for the investigation of the removal of BPA and NP from urban wastewater: wetlands were operated at HRT of 1.8 d and resulted in removal efficiencies of 73,, 70 and 62% of the influent 8.8 µg L^{-1} for the two planted with *Heliconia psitacorum* and *Phragmites australis*, and one non-planted CW, respectively. Changwichan (2007) stated that Cattail-based (*Typha* sp.) wetland had a capacity to remove 80% of BPA at HRT 10 d, though, based on our literature review, no data on the influent concentration was found (Papaevangelou et al., 2016). Pilot scale HSF-CWs for treatment of municipal wastewater, were also applied by Papaevangelou et al. 2016. The study indicated moderate removal efficiencies for BPA, at 49.6%, 50.0% and 55.4% for the mesocosms planted with *Phragmites australis*, *Typha latifolia* and the non-planted unit, respectively. Another pilot system consisted by two 0.65 m^2 HSF-CWs working in parallel, followed by a 1.65 m^2 HSF-CW in series and planted with *Phragmites australis*, was used for wastewater treatment from a mix of EOCs, including BPA (Ávila et al., 2010). At HRT of 3.5 d, the system demonstrated 70-90% removal of the 1.5 µg L^{-1} BPA influent concentration after the two small units and 85-99% at the final effluent. It is worth noting that in this study as well, granular medium of the wetland consisted by small-sized gravel. When Avila et al. (2013) investigated the role of redox on EOCs removal by HSF-CWs, redox indicated to play key role on the performance. Wetlands worked in different saturated conditions and results revealed significantly higher removal of BPA at the batch line (saturate/unsaturated phases), virtually 90%, compared to the control (settler-wetland permanently saturated) and also at the anaerobic line (79 and 65%, respectively).

Removal of antibiotics

In contrast with BPA, the impact of HRT on CIP concentration removal was not significantly different. Mean concentration removal was further increased only by 10%, after switching HRT to double (Table 18). Mass removal rate was calculated 1.5 and 0.7 mg h^{-1} at HR1 and HR2 treatments, respectively, difference that is attributed to the reasons mentioned above (case of BPA). As far as SMX concentration removal is concerned, despite the fact that statistical analysis showed significant difference with the change of HRT (p=0.008), the overall efficiency of the removal of the sulfonamide antibiotic was not satisfactory, as illustrated in Fig. 32, especially in the case of HRT 1 d, where negative removal was observed.

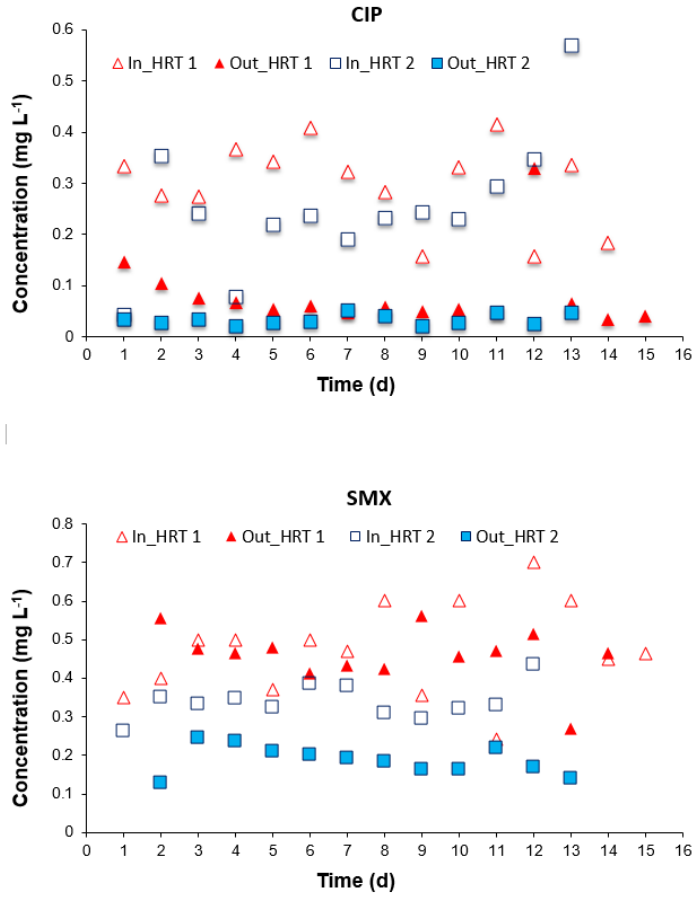


Figure 32. Comparison of the influent and effluent concentration of antibiotics versus time, with respect to different HRTs. Effluent concentrations have been corrected in respect to evapotranspiration.

SMX is generally characterized resistant to biodegradation (Sui et al., 2015). In this aspect, several authors have reported negative removal efficiencies of some organic compounds, especially from the group of sulfonamides including SMX. This finding is ascribed to deconjugation of conjugated metabolites during the treatment process (Gulkowska et al., 2008;

Choi et al., 2016; Verlicchi and Zambello, 2014; Radjenovi, 2008; Breitholtz et al., 2012). Gulkowska et al. (2008), studying the removal of nine antibiotics by WWTPs, indicate also two other approaches in this issue: firstly a possible adsorption of an amount of antibiotics onto the organic matter that is then being filtered out during filtration of the sample that leads to an underestimation of the actual concentration. A third possible scenario of this observation is the adsorption behavior of the analytes to particles, which might be changed during treatment, reflecting to the ratio among influent and effluent water (Lindberg et al., 2005). Additionally, reversible transformation of this group of antibiotics that lead to apparent higher concentrations at the effluent, is stated by Dan et al. (2013).

Impact of seasonality on BPA removal

In the present study, the effect of seasonality on BPA concentration removal was tested by the comparative evaluation of two experiments conducted during June (HR2 treatment) and December (OB treatment), under the same experimental conditions (HRT, influent concentration) (Fig. 34). Findings, indicated high removal efficiency in both treatments (Table 18), with a slightly better performance, as anticipated, in the warm period. Significance was tested by the independent sample t-test ($p < 0.05$) and bootstrap simulation, with 1000 iterations of analysis. To this respect, statistically significant difference was not found for BPA removal during the two experimental runs. Higher removal efficiencies of EDCs have been also measured in the summer period from Hijosa-Valsero et al. (2010), highlights that apart from the type of the organic substance and design criteria of the wetland, additional factors are of high importance for the removal of EOCs in HSF systems, such as sewage composition and environmental parameters (such as temperature, sunshine).

Temperature has great influence on the rate of biological and chemical processes in CWs, such as nitrification, denitrification and BOD_5 decomposition. High temperatures, promote evapotranspiration rate, which is directly associated with removal of organic contaminants with log K_{ow} between 0.5–1 and 3–3.5 (Garcia-Rodríguez et al., 2014), through plant uptake (Hijosa-Valsero et al., 2010b). In the well-documented review of Garcia-Rodríguez et al. (2014), seasonality and temperature are further discussed, as they directly influence plant and microbial growth, stating that in warm temperatures of 15-25 ^oC, optimal activity of the latter is achieved. However, the optimum temperature range for efficient growth of microbes is related to both their species and the growth medium (Vymazal and Kröpfelová, 2008). Thus, for mesophiles, optimum temperature is 30-45 ^oC, while obligate phycrophiles are grown better at the range of 15-18 ^oC (Shimp et al., 1993). In the warm climate of Crete, average temperatures of experimental runs HR1 and HR2 well exceeded 25 ^oC, while average temperature of cold season measured not far from the down limit of 15 ^oC, as noted by Garcia-Rodríguez et al., (2014) (Table 15). In conclusion, Matamoros et al. (2016) tested different WWTP technologies

for EOCs treatment from wastewater. Among these, they were included two gravel based HSF-CWs of 600 and 1000 m^2, planted with *Phragmites australis* and working intermittently at HRT of 4-6 days. Statistical analysis of the results, demonstrated the dependence of EOCs removal on seasonality, with efficiencies of 24 and 49% between the cold and warm season respectively, probably due to the higher activity of plant roots and the greater biofilm in the gravel bed, in warm season. In another study investigating pesticides removal with CWs planted with macrophytes, lower efficiency was revealed in winter than in summer, pointing out the strong contribution of a pesticide removal with evapotranspiration in summer (Lv et al., 2016).

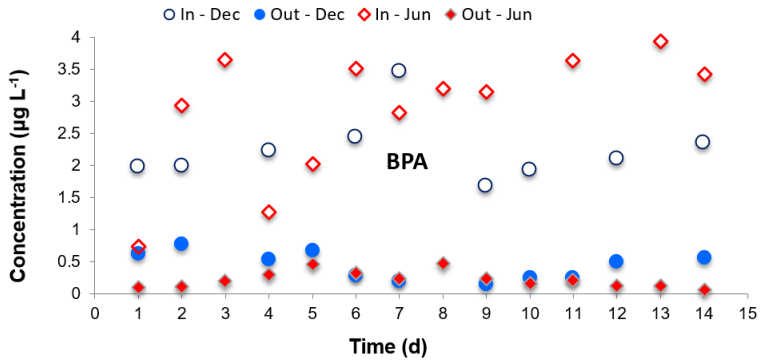


Figure 33. Comparison of the influent and effluent concentration of BPA versus time, during June and December. Effluent concentrations have been corrected in respect to evapotranspiration.

Secondary treatment

Operation of CWs as an alternative secondary treatment technology for the removal of EOCs from sewage, emerges growing concern in several countries such as Italy, Portugal, Spain, Denmark, China, Canada and the USA (Li et al., 2014b). In the present research, a preliminary study took place for the investigation of systems response on feeding with primary treated wastewater, with no spiked contaminants. Results indicated removal attenuation of the average influent BPA concentration of 0.9 µg L^{-1} to a measured concentration of 0.7 µg L^{-1}, that after adjustment in respect to evapotranspiration corresponded to approximately 50% removal (Table 18; Fig. 35). Mass removal rate was found to be the lowest among all treatments, equal to 1.5 µg h^{-1}. The percentage removal of the removed mass over the mass entering the system was calculated 54%.

Nonetheless, it was evident that matrix effect of primary treated wastewater, worsen the reliability of the detection method. Antibiotics could not be detected without pre-concentration of the samples, a key process that apart from dealing with interferences through sample clean-up, also offers the possibility for detection of much lower concentrations (Seifrtová et al., 2009).

We are aware that our research may have not addressed this limitation, thus we did not investigate the fate of antibiotics during depuration by HSF-CW, though this was only a first insight of the technology involved and remains an ongoing research study.

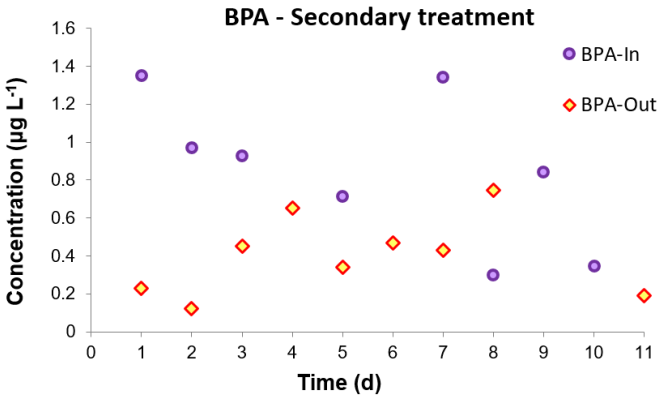


Figure 34. BPA concentration at the influent and the effluent of the HSF-CW, during PR treatment, i.e. feeding with primary treated wastewater.

HSF-CWs are among the most commonly used types of artificial systems that use plants for secondary wastewater remediation (Zhang et al., 2014b). Notwithstanding, surface flow, vertical flow and hybrid systems have been also applied against PPCPs with satisfactory results, comparable or even greater than those of conventional treatment systems (Hijosa-Valsero et al.2016; Verlicchi and Zambello, 2014). An explanation for this could be the co-existence of both aerobic and anaerobic processes in CWs, whereas, lack of heterogeneous conditions in WWTPs elicit fewer degradation pathways (Zhang et al., 2014a). Consequently, results so far regarding application of CW systems for depuration of PPCPs and EOCs in general, from primary treated effluents, are promising and should be validated by a wider range of experiments.

Table 17. The impact of different operating conditions as reflected by the results of statistical analysis of paired treatments: control (C) – T, HR1 – HR2 and HR1 – OB ($p < 0.05$).

	Parameter	Paired treatments	P-value	Significant difference
BPA	Vegetation	C-T	0.046	YES
	HRT	HR1-HR2	0.049	YES
	Seasonality	HR1-OB	0.099	NO
CIP	Vegetation	C-T	0.004	YES
	HRT	HR1-HR2	0.360	NO
SMX	Vegetation	C-T	0.074	NO
	HRT	HR1-HR2	0.008	YES

Table 18. Overall efficiency of the vegetated constructed wetland, in terms of the impact of HRT, seasonality and as a secondary wastewater treatment. C_{in} and C_{out}^{ET} are the mean measured concentrations at the influent and the effluent of the wetlands (12<n<13). ET indicates that corresponded values have been corrected in respect to evapotranspiration. m_p is the removed mass of the contaminant over the mass entering the system. In the parenthesis is p-value, with significance level set at p < 0.05.

Organic compound	Treatment	HRT (days)	Measured influent concentr. BPA (μg L^{-1}) CIP, SMX (mg L^{-1})	Measured effluent concentr. BPA (μg L^{-1}) CIP, SMX (mg L^{-1})	Concentration removal (%)	Concentration removal ET adjusted (%)	Significant difference (C_{in}-C_{out}^{ET})	Rate of mass removal ET adjusted BPA (μg h^{-1}) CIP, SMX (mg L^{-1})	m_p (%)
BPA	HR1	1	4.1	2.5	38.5	48.3	Yes (0.032)	12.8	48.3
	HR2	2	3.1	0.4	86.9	92.2	Yes (0.022)	9.4	92.3
	PR	2	0.9	0.6	24.7	52.6	No (0.156)	1.5	53.5
	OB	2	2.2	0.6	71.7	80.8	Yes (0.008)	5.9	80.8
CIP	HR1	1	0.30	0.1	72.4	76.8	Yes (0.001)	1.5	76.9
	HR2	2	0.25	0.1	77.5	86.7	Yes (0.006)	0.7	86.7
	PR	2	< D.L.	-	-	-	-	-	-
SMX	HR1	1	0.47	0.55	-15.3	3.2	No (0.460)	-0.3	3.1
	HR2	2	0.54	0.32	6.5	44.8	Yes (0.180)	0.5	65.6
	PR	2	< D.L.	-	-	-	-	-	-

5.4.4. Physicochemical characteristics of municipal wastewater

It has been demonstrated that physical and chemical phenomena, such as adsorption (at the root surface, on the organic matter or at the produced biofilm) may be affected among others, by the type of materials that the substrate is composed of and physicochemical parameters, such as pH, temperature, redox conditions (Hijosa-Valsero et al., 2010; Dordio and Carvalho, 2013). Apart from adsorption and in general the abiotic processes that are involved in the removal of organic pollutants in CWs, physicochemical parameters influence living organisms such as plants and microorganisms, hence, biodegradation pathways of the organic compounds (Hijosa-Valsero et al., 2010b). Moreover, physicochemical parameters are remarkably influenced by the flow type and the flow conditions (FWS vs SSF CWs) (Zhang et al., 2014b). In this regard, as well as for an overall evaluation of the HSF-CW, water quality parameters and nutrients were measured along with the organics, during different operational phases of the system (Table 20 and Fig. 36-38). Physicochemical parameters of wastewater at the influent and effluent of municipal WWTP of Chania are shown in Table 19. Results are sorted by date and presented separately in groups, according to i) the CW tested (control vs *J. acutus*), ii) whether addition of organic contaminants was conducted or not (the latter named as "no spike") and iii) the applied HRT.

Table 19. Mean and maximum concentrations of water quality parameters at the effluent of the WWTP of the city of Chania for 2015 (in mg L^{-1}). Number of samples is indicated with "n".

	mean	max	n
BOD_5	9	48	115
COD	22	70	47
SS	5	40	210
TN	10	23.5	50
NH_4-N	1.9	12.3	54
TP	6.9	9	37

Results obtained from http://www.deyax.org.gr/

In the present study, BOD ranged between 56 and 61% at HRT 1 d and 1.4 d respectively. Though, influent concentration in the first treatment was much higher than in the second (36.5 and 9 mg L^{-1}). With regard to the planted mesocosm, removals exceeded 66% and reached 91% in the non-spiked treatment (J3), where BOD at the influent was measured 27.5 mg L^{-1}. Secondary treated wastewater that was primarily applied to the system, had low BOD values. However it is important to notice that spiking yielded a further increase of BOD and COD values, not only due to the addition of the organic substances, but also because of the solvents used for the dilution of the compounds. It was calculated that solvents did not exceed the level

of 0.5 ml L^{-1} at the total influent wastewater. This phenomenon was more evident in experiment T, in which higher concentrations were applied. BOD analysis was conducted for five treatments (2 x control and 3 x vegetated treatments) and afterwards, only COD analysis was performed until the end of the experiments. However, due to experimental failure, results were not considered reliable, hence are not presented among the results.

In the literature review concerning the application of CWs for wastewater treatment (Zhang et al., 2015), a comparison in terms of the efficiency of HSF-CWs to treat mainly municipal wastewater, is compiled. An average removal of BOD at 75% and COD at 66% is reported, for average effluent concentrations of 20.3 and 65 mg L^{-1}, respectively. Significantly increased reduction in BOD with the presence of plants, was reported by Upadhyay et al. (2016), with both HRTs of 12 and 24 h. In the research study of Akratos and Tsihrintzis (2007), design parameters for the removal efficiency of five pilot-scale HSF-CWs, was investigated: mesocosms planted with *Phragmites australis* and *Typha latifolia*, exhibited BOD removal of the influent 41 and 54 mg L^{-1}, at 88 and 85%, respectively, whereas in the absence of plants, the removal of 50 mg L^{-1} slightly differed from the planted units (86%). Similarly, high performance was addressed regarding COD removal. Influent concentrations of 62–89 mg L^{-1} were reduced more than 85% in all wetlands tested, without exhibiting any influence of vegetation on the removal.

Electrical conductivity (EC) is a useful indicator of salinity or total salt content. Salinity of CWs is an important factor because it directly affects wetland environment (Dong, 2012). Moreover, salinity inversely affects solubility of dissolved oxygen (Fondriest Environmental, 2014). The overall results demonstrated that EC had an increasing trend in the effluents, especially in high HRT (2 days). Increased EC at the effluent of the CWs has been reported by several authors (Akratos and Tsihrintzis, 2007; Weerakoon et al., 2013; Kelvin and Tole, 2011; Caselles-Osorio and Garcia, 2007; Monsalvo, 2015), though the opposite is also frequently addressed (Papaevangelou et al., 2016) primarily for HRTs > 10 d (Monsalvo, 2015). This increase, could be attributed to the evapotranspiration (Caselles-Osorio and Garcia, 2007) and the depth of column, since the increase of depth has been reported to induce higher EC values (Dong, 2012).

In this study, the impact of HRT on the increase of EC was evident. Influent EC in the range of 908-1349 μS cm^{-1}, was increased at 14-21% at the effluent, with HRT of 1 day and more than 46% at a HRT of 2 days, in the vegetated mesocosm. In the run with primary wastewater supply (PR treatment), the highest value of 2475 μS cm^{-1} was measured at the effluent. In a SSF-CW fed with primary settled wastewater, EC was slightly higher in the effluents up to values of 2000-2800 μS cm^{-1} (Caselles-Osorio and Garcia, 2007). On the other hand, in the study of

Papaevangelou et al. (2016), EC was decreased at the effluent: from 1638 to 1192 μS cm^{-1} at a HSF planted with *P. australis*, from 1224 to 1195 μS cm^{-1} at a HSF planted with *Typha latifolia* and from 1094 to 1215 μS cm^{-1} at the non – vegetated wetland.

Total suspended solids (TSS) are removed by several processes in CWs, such as filtration, adsorption onto the biofilms (developed in the gravel particles and root surface), precipitation, microbial assimilation, flocculation and sedimentation (Upadhyay et al., 2016; Sultana, 2014). The relatively low flow rate in SSF systems, provide conditions for adequate TSS removal, acting like horizontal gravel filters (Brown et al., 2000). The impact of vegetation on the removal of TSS, is often doubted, since no significant differences are reported in various studies (Liehr et al., 2000).

Concerning the efficiency of the HSF of the present study, in terms of TSS removal, significant oscillations at the influent concentration were displayed: in the control mesocosm ranged from 9.5 to 56 mg L^{-1}, probably mainly caused by the growth of photosynthetic (green) thread algae around the submerged pump. Though, treatment C3 (Table 20) far exceeded this range because of major equipment failure in the WWTP (TSS: 129 mg L^{-1}, S.E.: 63). Moreover, in treatment PR (J8) influent TSS concentration was, as anticipated, increased (72 mg L^{-1}). In compared experimental runs C.1 with J.3 (Table 20), conducted under the same HRT (1 d) and similar influent concentration of TSS (47.7 and 56.5 mg L^{-1}, respectively), a removal efficiency of 91% was obtained for the vegetated and 83% for the control wetland.

The above results are reasonable, based on previous studies. In the afore-mentioned review of Zhang et al. (2014b), a mean effluent concentration of TSS after treatment using HSF-CWs was approximately 20 mg L^{-1}, corresponding to removal efficiency close to 80%. In the recent study of Upadhyay et al. (2016), 80% removal was obtained using HSF-CWs planted with *Potamogeton crispus* and *Hydrilla verticillata* macrophytes. TSS removal at a range of 59.7-98.8% was also reported by Akinbile et al. (2012). Another review on CWs, cited treatment efficiency at 77% of the 141 mg L^{-1} influent concentration, with HSF systems (Vymazal, 2010).

In general, pH is a critical factor because it affects microbial structure and activity and moreover, organic matter solubility, nutrient uptake and availability of nutrients and metals from soil or other wetland substrates (Paranychianakis et al., 2006; Fondriest Environmental, 2013). Alkaline pH could also provide optimal condititions for nitrification or other special biochemical processes (Vymazal, 2007; Zhang et al., 2010). In the present work, the average pH values did not reveal any significant difference, neither between the influent and the effluent, nor among the planted and non – planted wetlands. However, slightly lower pH values were observed at the effluents. This could be attributed to the organic acids produced by the roots of *J. acutus* (Bais et al., 2006; Paranychianakis et al., 2016).The mean influent pH values

in the planted mesocosm were 7.3-7.7, the latter in primary treated influent (PR treatment). The pH values in the vegetated wetland decreased from 0.1 to 0.3 units at HRT of 1 day and 0.3 to 0.6 units at HRT of 2 days, respectively. Not significant effect between non – planted and planted with common reeds HSF-CWs, was also noted by Zhang et al. (2010). Notwithstanding, in the study of Upadhyay et al. (2016), who examined the performance of a three stages CW, the HSF section, planted with *P. crispus* and *H. verticillata*, yielded to a decrease in pH from 7.3 to 6.4 at HRT of 2 days. pH dropped from 7.2 to 7.1 at HRT of 1 day. Results exhibited significant difference ($p < 0.05$) only for the vegetated unit, operating at HRT of 2 days. Correspondingly, Lv et al. (2016) reported significantly lower effluent pH values in all planted mesocosms, and conversely significantly increased pH in non-planted ones.

Table 20. Mean concentrations with standard errors (S.E.) for the influent and effluent of the two wetlands (control: C1, 2 and *Juncus:* J1-10) for water quality parameters. "Spike" and "no spike" indicates the addition or not of the organic contaminants.

C.1 CONTROL, HRT 1 d, (n=4)	Influent		Effluent		NO spike
1-14/12/15	mean	S.E.	mean	S.E.	reduction (%)
pH	7.5	0.1	7.6	0.0	-0.8
BOD (mg/L)	36.5	12.4	16.0	4.3	56.2
COD (mg/L)	52.3	11.6	42.1	7.0	19.4
Conductivity (µS/cm)	1033.5	32.2	1158.0	24.1	-12.0
TSS (mg/L)	47.7	24.0	8.1	2.4	83.0

C.2 CONTROL, HRT 1.4, d (n= 5)	Influent		Effluent		NO spike
23-29/1/16	mean	S.E.	mean	S.E.	reduction (%)
pH	7.1	0.04	7.3	0.06	-2.8
BOD (mg/L)	9.0	0.5	3.5	1.5	61.1
COD (mg/L)	17.1	4.5	14.1	5.2	17.7
Conductivity (µS/cm)	913.0	22.5	975.2	21.9	-6.8
TSS (mg/L)	9.5	3.0	2.4	0.9	74.3

J1. HRT 2d (n= 13), spike	Influent		Effluent		
23/5 - 19/9/14	mean	S.E.	mean	S.E.	reduction (%)
pH	7.6	0.06	7.3	0.07	3.5
BOD (mg/L)	52.6	8.7	17.9	3.9	66.0
Conductivity (µS/cm)	1349.4	64.3	1634.0	68.2	-21.1
TSS (mg/L)	13.9	2.9	7.0	1.9	50.1

J2. HRT 1d (n=11)	Influent		Effluent		
4/5-29/7/15	mean	S.E.	mean	S.E.	reduction (%)
pH	7.5	0.04	7.3	0.04	3.0
BOD (mg/L)	42.8	7.1	10.1	2.0	76.3
Conductivity (µS/cm)	1138.3	24.1	1381.0	32.3	-21.3
TSS (mg/L)	13.6	1.5	4.0	0.8	70.9

J3. HRT 1 d, No spike	Influent		Effluent		
1/12/15-29/1/16	mean	S.E.	mean	S.E.	reduction (%)
pH	7.3	0.1	7.1	0.07	2.4
BOD (mg/L)	27.5	8.06	2.5	1.32	90.9
COD (mg/L)	53.9	28.1	12.1	3.95	77.5
Conductivity (µS/cm)	1077.6	176.1	1240.0	76.4	-15.1
TSS (mg/L)	56.5	26.4	5.1	1.35	91.0

J4. Treatm. T, HRT 1d (n=5)	Influent		Effluent		
7-19/3/16	mean	S.E.	mean	S.E.	reduction (%)
pH	7.4	0.1	7.3	0.1	0.5
COD (mg/L)	651.3	122.4	473.0	78.9	27.4
Conductivity (µS/cm)	1031.0	68.1	1194.3	39.3	-15.8
TSS (mg/L)	31.2	26.9	9.3	3.5	70.3

Continue of Table 20.

J5. HRT 1 d, No spike	Influent		Effluent		
21/3/16-10/4/16	**mean**	**S.E.**	**mean**	**S.E.**	**reduction (%)**
pH	7.4	0.09	7.3	0.12	2.3
COD (mg/L)	18.0	1.15	12.7	0.88	29.6
Conductivity (µS/cm)	907.8	57.64	1035.0	84.13	-14.0
TSS (mg/L)	2.5	0.39	3.9	3.09	-56.1

J6. Treatm. HR1, HRT 1d (n=5)	Influent		Effluent		
14-28/4/16	**mean**	**S.E.**	**mean**	**S.E.**	**reduction (%)**
pH	7.37	0.14	7.14	0.14	3.1
COD (mg/L)	75	9.93	49	6.86	34.7
Conductivity (µS/cm)	1054.6	29.96	1202.8	35.13	-14.1
TSS (mg/L)	10.1	1.59	9.1	1.78	10.7

J7. Treatm. HR2,HRT 2d (n=5)	Influent		Effluent		
28/5-10/6/16	**mean**	**S.E.**	**mean**	**S.E.**	**reduction (%)**
pH	7.74	0.04	7.41	0.04	4.3
COD (mg/L)	32	8.29	21	3.47	35.2
Conductivity (µS/cm)	1009	11.88	1965	107.77	-94.8
TSS (mg/L)	9.3	1.40	2.5	0.38	73.5

J8. Treatm. PR2, HRT 2d (n=8)	Influent		Effluent		
11-27/7/16	**mean**	**S.E.**	**mean**	**S.E.**	**reduction (%)**
pH	7.74	0.02	7.1	0.02	8
Conductivity (µS/cm)	1541	14	2475.5	108.41	-61
TSS (mg/L)	71.26	9.52	33.60	9.77	53

J.9 HRT 1d (n=5), spike	Influent		Effluent		
7-17/9/16	**mean**	**S.E.**	**mean**	**S.E.**	**reduction (%)**
pH	7.51	0.03	7.21	0.03	3.9
Conductivity (µS/cm)	1114	12	1316	12	-18.1
TSS (mg/L)	10.73	4.44	5.62	2.72	47.57

J10. OB, HRT 2d	Influent		Effluent		
4/11-16/12/16	**mean**	**S.E.**	**mean**	**S.E.**	**reduction (%)**
pH	7.62	0.05	7.08	0.05	7.0
Conductivity (µS/cm)	1206	65.82	1763	65.82	-46.2
TSS (mg/L)	17.9	7.45	2.7	0.58	85.0

Nutrients removal

Total nitrogen (TN) includes organic nitrogen, nitrate, ammonium and nitrite. Several processes are related with the removal of nitrogenous compounds, namely, nitrification, denitrification, ammonification, ammonia volatilization, plant uptake and adsorption (Leung et al., 2016). Nitrification could be enhanced in aerobic conditions due to proliferation of the nitrifying and the ammonia-oxidizing bacteria (Leung et al., 2016; Vymazal, 2007)

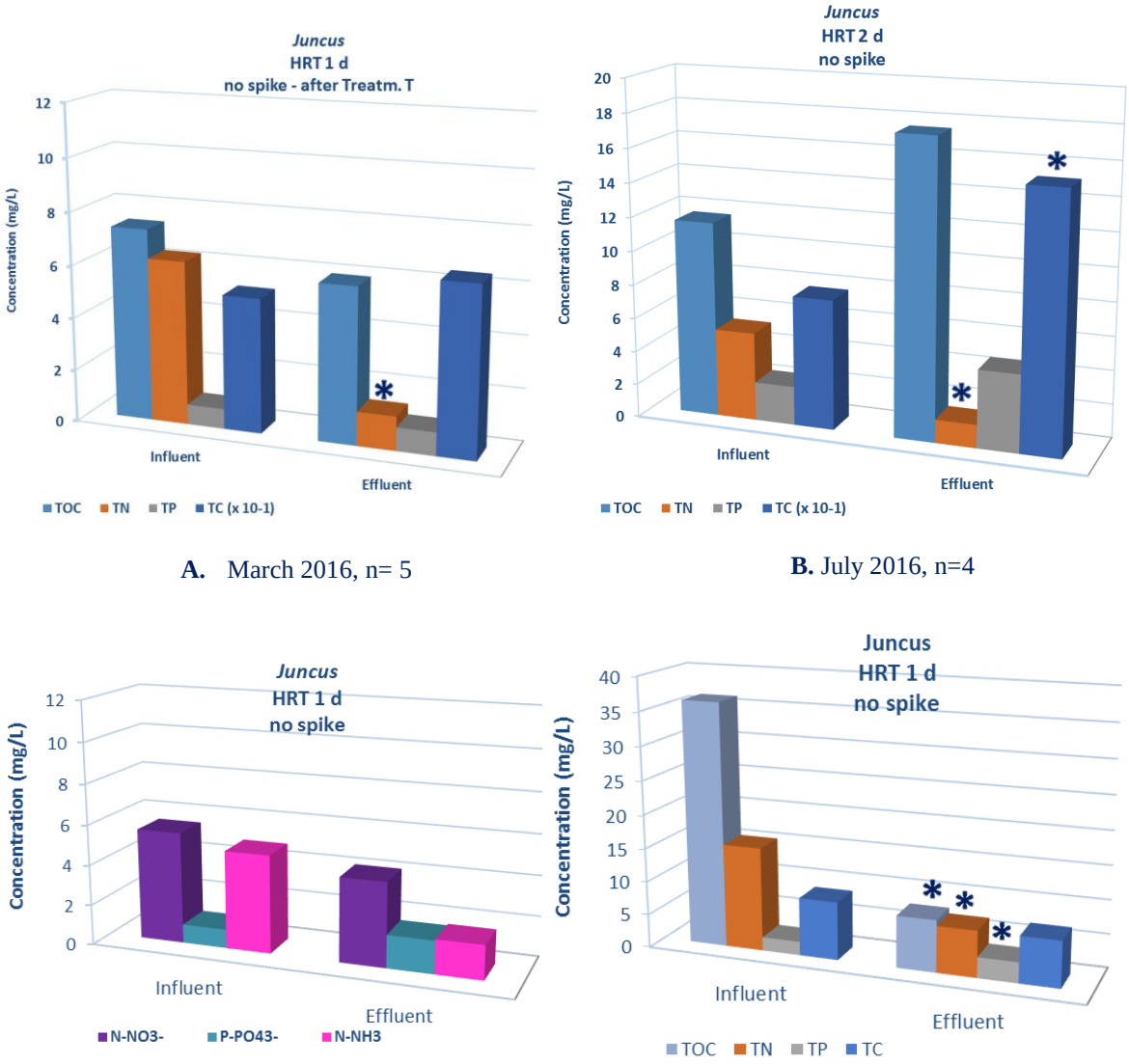
In this study, the vegetated CW was able to significantly decrease TN concentration in all treatments, except for PR (Fig. 36, 37). In the absence of plants, TN slightly decreased, but with no statistically significant differences (Fig. 38). However, greater removal of nitrogen and phosphorus by HSF-CWs could be achieved with longer HRTs (Akratos and Tsihrintzis, 2007). Our results are in conformity with several other comparative studies, that indicated the positive effect of vegetation to TN removal (Lv et al., 2016; Paranychianakis et al., 2016; Stecher and Weaver, 2003; Villaseñor Camacho et al., 2007).

Removal of phosphorus is strongly related to physical processes, such as adsorption, precipitation, filtration and accumulation in the organic matter or substrate. Additional mechanisms involved are sedimentation along with suspended solids, microbial activity and plant uptake. In particular for HSF systems, precipitation is appeared to have secondary role in phosphorus removal, with an average removal ranging between 40-60% (Vymazal, 2007). In this work, a slight trend for TP concentration to be increased at the effluent was not statistically verified, apart from PR treatment (Fig. 36, 37). In most studies dealing with CWs, TP concentration is found to be decreased at the effluent (Akratos and Tsihrintzis, 2007). Although, HSF systems are characterized as not effective at TP removal, due to the limited adsorption of phosphorus to the gravel or stone bed commonly used, because of the low concentrations of Ca, Fe or Al (Vymazal, 2004). Removal of phosphorus in the CWs could be attributed to the assimilation of nutrients by *J. acutus* plants, the photosynthetic rate, and pH that mediated co-precipitation of phosphorus with $CaCO_3$ (Upadhyay et al., 2016). Besides, other authors described the opposite trend, thus an increase of TP concentration at the effluent.

Reduction of the total organic carbon (TOC) was measured in the majority of treatments tested, both in planted and control pilots. The recorded TOC, reflected the high organic loading during PR and all spiked treatments, with maximum TOC measured in the influent, at 35 mg L^{-1} (Fig. 37: D, Fig. 36: C). Highest removal of TOC at 78% of the initial 36.2 mg L^{-1}, was obtained for the non – spiked treatment, ran on January 2016, as illustrated in Fig. 36: C. Furthermore, in the literature has been depicted that low temperatures lead to low removal of TOC from wastewater (Jakubaszek and Sadecka, 2015).

Nitrate nitrogen (NO_3-N) concentration at the influent and effluent of the vegetated wetland, exhibit a statistically significant difference during operation with HRT at 2 days (Fig. 37: E) and at the first of control treatments (Fig. 38: C1). Ammonia nitrogen concentration (NH_3-N) was decreased at the effluent of all the vegetated treatments, although no significance was indicated. On the other hand, in control, treatments the opposite trend was occurred, but statistical analysis did not result in significance neither. Free orthophosphate (PO_4^{3-}) is primarily removed by adsorption and plant uptake and it is the only form of phosphorus, considered to be utilized directly by the macrophytes. Therefore, constitutes a key link between organic and inorganic phosphorus cycling in wetlands (Akratos and Tsihrintzis, 2007; Vymazal, 2007). Regarding P-PO_4^{3-} concentration, no statistically significant removal was obtained, apart from a control treatment that found to be increased from 2.00 to 3.64 mg L^{-1}.

Juncus acutus CW – No spike



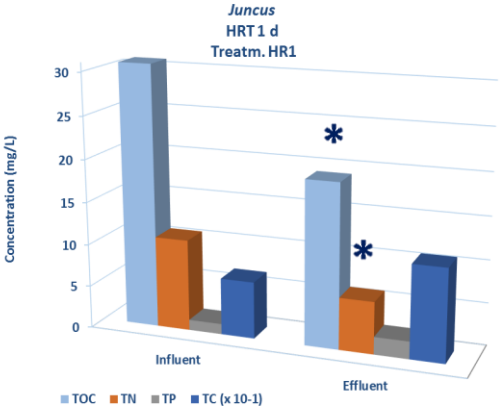
A. March 2016, n= 5

B. July 2016, n=4

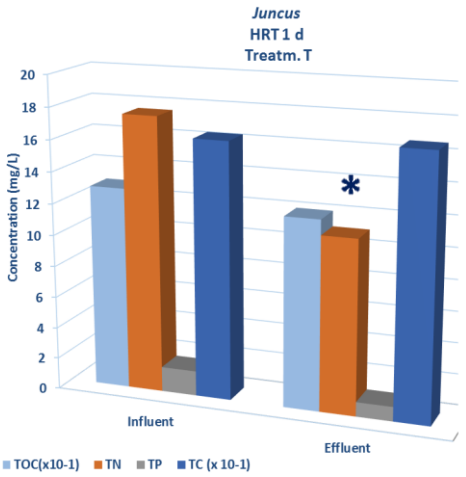
C. January 2016, n=10

Figure 35. Concentrations of $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$, $N\text{-}NH_3$ (bottom left) and TOC, TN, TP, TC (right) at the influent and effluent of the vegetated wetland. During treatments no organic contaminants was spiked to the system. Number of samples is indicated with "n". Statistical significant difference between influent and effluent concentrations is indicated with a star (*).

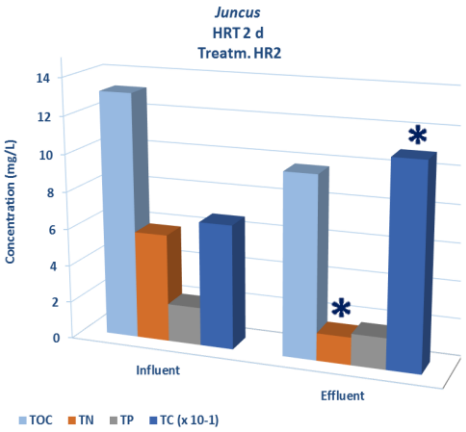
Juncus acutus CW + Organics



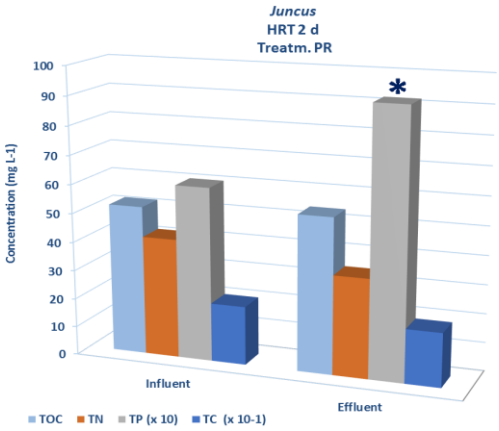
A. HR1 - April 2016



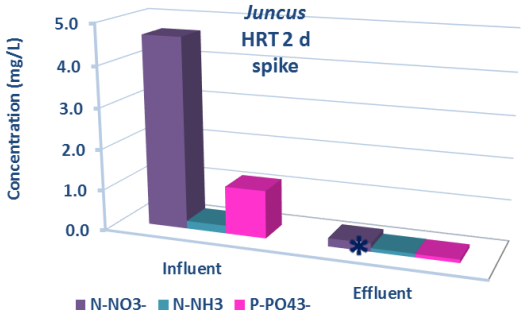
B. HR2 - March 2016



C. HR2 - June 2016



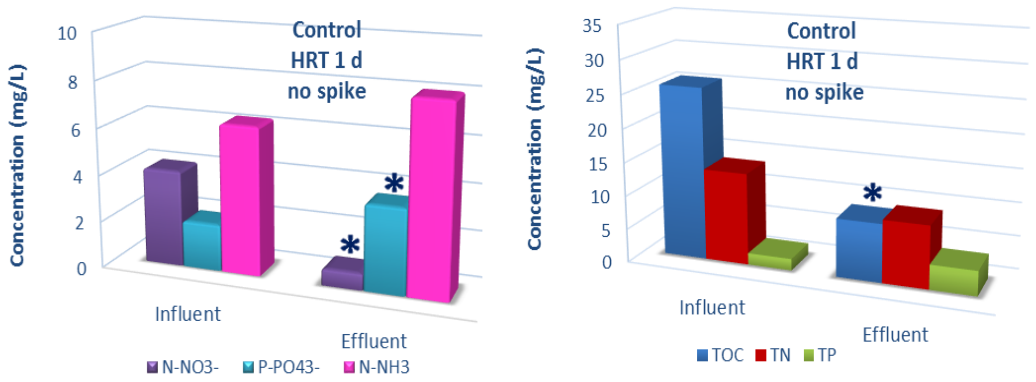
D. PR - July 2016



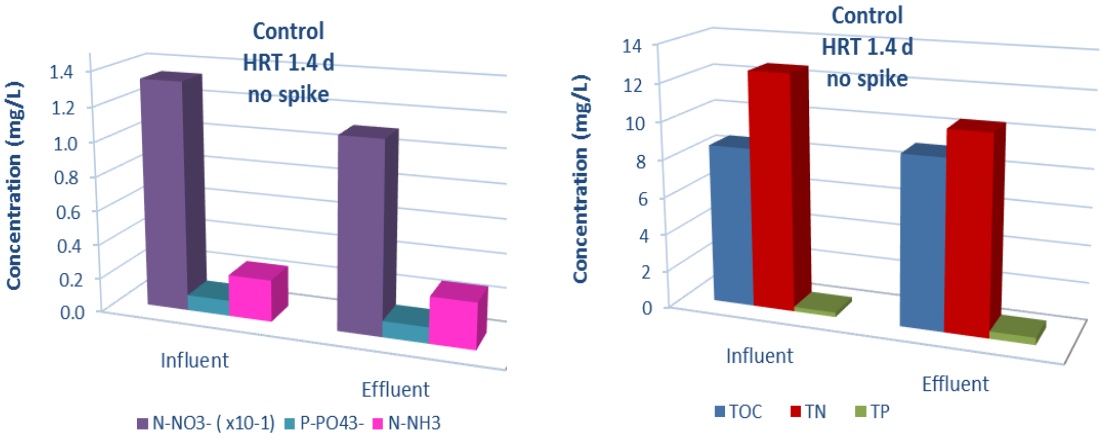
E. BPA spike - September 2015

Figure 36. Concentrations of TOC, TN and TP at the influent and effluent of the vegetated wetland, during treatments: T, HR1, HR2 and PR (A to D). Concentrations of $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$, $N\text{-}NH_3$ at the influent and effluent of the vegetated wetland, during spiking with BPA (E). Number of samples is indicated with "n". Statistical significant difference between influent and effluent concentration is indicated with a star (*). A, B, C, E: n=5, D: n=8.

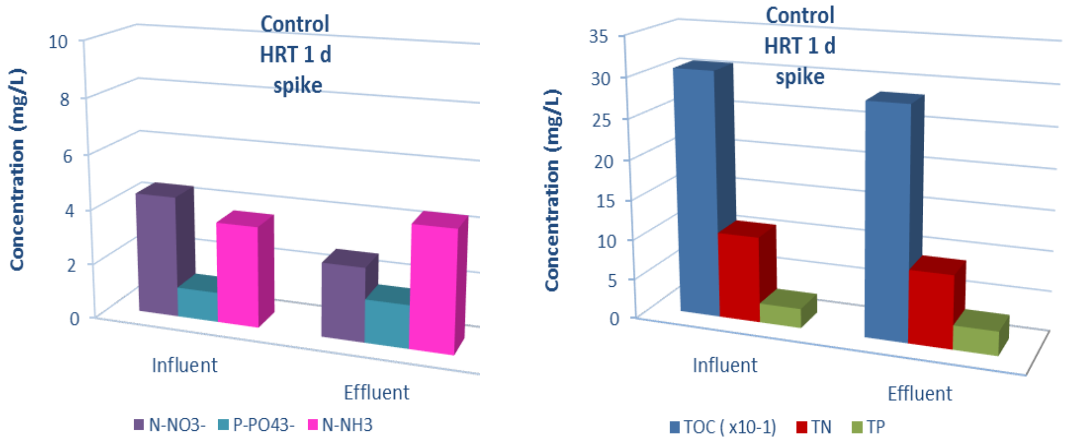
Control CW – No vegetation



C1: December 2015, n=5



C2: January 2016, n=5



C3: January 2016, n=5

Figure 37. Concentrations of $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$, $N\text{-}NH_3$ (left) and concentrations of TOC, TN and TP (right) at the influent and effluent of the non-vegetated wetland. The addition of organic contaminants is indicated as "spike" and no addition as "no spike". Number of samples is indicated with "n". Statistical significant difference between influent and effluent concentration is indicated with a star (*). Treatments C1, C2, C3 are referred to Table 20.

5.4.5. Concluding remarks

In the present study, the performance of a horizontal subsurface flow constructed wetland, for the remediation of municipal wastewater from EOCs, was evaluated. The CW was planted with five *Juncus acutus* halophytes and wastewater was spiked with bisphenol A, ciprofloxacin and sulfamethoxazole.

Analysis of the results demonstrated the contribution of plants on BPA removal. Concerning the first phase of the experiments, CW was able to remove 75 – 77% of the 5 μg L^{-1} influent concentration of the endocrine disruptor, throughout two experimental runs of 50 and 41 days, respectively. In the second phase of the experimental procedure, testing the efficiency of the system to treat co-contaminated secondary treated municipal wastewater, several operating parameters where investigated. Investigation of the impact of vegetation was carried out through the comparison of a planted and a non-planted wetland. The ratio of the removed BPA mass, to the BPA mass entering the system, was estimated to be 73% in the vegetated wetland, compared to 27% in the absence of vegetation. CIP concentration was significantly decreased in the control unit, addressing biodegradation in the absence of plants. Nonetheless, contribution of vegetation was reflected with a further increase in the rate of mass removal in the vegetated wetland, by 30%. Performance of the system in terms of SMX removal was significantly lower and results unstable, though consistent with previous studies, indicated complex behavior and incomplete removal.

Concerning the impact of hydraulic retention time, the present study confirmed the high sensitivity of the systems performance in terms of BPA removal, with changes of HRT, corroborating similar other studies. Superior treatment performance at longer HRT is displayed by the significantly higher mass removal rate compared to short HRT (92 versus 48%, respectively). The impact of HRT on CIP removal was less important, while SMX removal ranged from low to moderate levels. Seasonality seems to enhance systems performance in terms of BPA removal, yielding 10% higher mass removal ratio in hot season. The HSF-CW system responded satisfactorily during operation as a secondary treatment system, however, removal performance was decreased.

Unfortunately, it was not possible, a non-planted system with the same design parameters with the vegetated one, to be installed and run in parallel during the entire experimental period, in order to provide more comparative data. Though, control CW used in this study, contributed significantly to the interpretation of the results. In order of a plant species to be appropriate for CW applications, several criteria should be met. For instance, tolerance to contaminated wastewater, high biomass production and deep and extensive root system to support

rhizosphere effect (Thijs et al., 2017) *Juncus acutus* plants obtained no visible toxicity symptoms, exhibited fast growth rate and intense rhizome propagation, and their leaves retained erected and green throughout the experimental period. Overall *J. acutus* plants exhibited efficient performance as a single plant species of the CW for municipal wastewater treatment.

5.4. CW with halophytes for the degradation of BPA in primary-treated municipal wastewater

Characterization of wastewater based on water quality parameters, as well as BPA measured in the influent and the effluent, are given in Table 21, in terms of minimum and maximum values for all the examined period (March – December 2013). As noted in the experimental design, the HSF-CW was connected in series with the FWS-CW that worked as a primary treatment wetland. However, some very high values showed in the table are due to bypass of the FWS after the end of November, causing an increase of the average inlet COD concentration that reached 250 mg L^{-1}, from the 100-120 mg L^{-1} that was before the change.

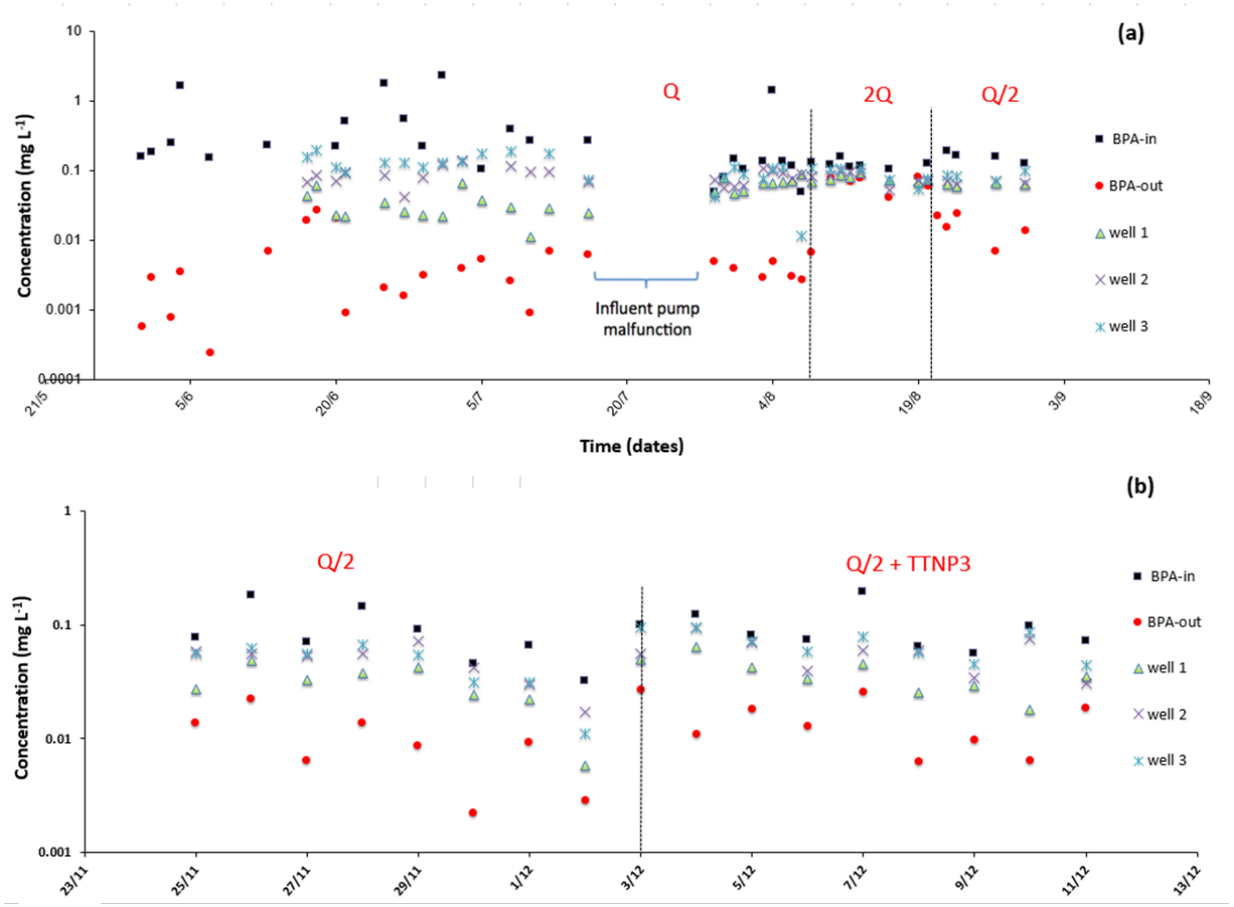
Table 21. Wastewater characterization

Parameter	Inlet	Outlet	
BPA	0.03 – 1.61	ND – 0.08	mg L^{-1}
pH	6.8 – 8.1	7.1 – 8.6	
EC	1.0 – 2.5	1.1 – 2.6	mS cm^{-1}
BOD	45-195	30-70	mg L^{-1}
COD	98.5 – 428.0	79.7 – 162.0	mg L^{-1}
Total Nitrogen	41.9 – 156.0	35.3 – 111.0	mg L^{-1}
Ammonium-N	10.9 – 86.5	10.1 – 61.1	mg L^{-1}
Nitrate-N	0.2 – 22.8	0.2 – 10.7	mg L^{-1}
Total Phosphorus	3.3 – 87.3	1.1 – 85.5	mg L^{-1}
Boron	0.2 – 0.6	0.01 – 0.4	mg L^{-1}

5.5.1. BPA removal

During the first period of operation of the CW, May to August 2013, the inlet flowrate was 1.41 m^3 d^{-1} (HRT = 1.80 d) and resulted in a significant reduction of 97% of the BPA concentration in the outlet as shown in Fig. 38 and Table 21. During this period, the inlet BOD was relatively low due to pre-treatment by the FWS constructed wetland (shown in Fig. 39) and it was in the range of 40-60 mg L^{-1}; while the COD ranged from 100 to 120 mg L^{-1}. The BPA measured in the inlet remained close to the nominal value of 0.15 mg L^{-1} as shown in Table 21.

Figure 38. (a) Bisphenol A concentrations in the influent, effluent and the three sampling wells of the horizontal subsurface flow system during the period May 31–September 1, 2013. (b) Bisphenol A concentrations in the influent, effluent and the three sampling wells of the HSF system during the period May 31–August 8, 2013 (Kalogerakis and Christofilopoulos, 2015).



The observed variations were due to non-perfect dissolution of BPA in tap water in the BPA reservoir. The CW was not operating for 13 days due to pump failure in the inlet of the CW. From the end of July 2013, the inlet flowrate was kept at its nominal value of 1.41 $m^3 d^{-1}$ for a period of 8 days. During this period BPA removal was also very high, reaching 90%. Subsequently, the inlet flowrate was doubled to 2.82 $m^3 d^{-1}$ corresponding to an HRT of 0.90 d. During this period the BPA removal dropped significantly to about 55%. The inlet COD and BOD decreased reaching their previously reported value. After two weeks of operation at double the nominal flowrate, the inlet flowrate was reduced to half its nominal value, 0.74 $m^3 d^{-1}$, corresponding to an HRT of 3.43 d. The operation of the CW lasted only a few days during which BPA removal was increasing. However, a major pump failure occurred and the primary-treated wastewater from the WWTP of the city of Heraklion could not reach the pilot. The CW remained inoperative until mid-November. The prolonged period was also due to renovations at the WWTP where the primary settling tank was renovated and relocated.

Table 22. Overall performance of constructed wetland regarding BPA removal. Mean, minimum and maximum concentrations at the influent and the effluent of the HSF-CW are given in mg L^{-1} the standard error of the means.

Treatment	Influent				Effluent				Average Removal (%)
	Mean	S.E.	Min	Max	Mean	S.E.	Min	Max	
Q (5/5 – 31/12)	0.332	0.09	0.048	1.615	0. 006	0.001	ND	0.028	97.5
2Q (7/8 – 19/8)	0.113	0.01	0.048	0.158	0.051	0.013	0.003	0.081	54.9
Q/2 (20/8 – 30/8)	0.095	0.013	0.031	0.190	0.016	0.004	0.001	0.060	85.2
Q/2 (25/11 – 2/12)	0.088	0.018	0.032	0.180	0.011	0.002	0.002	0.022	88.7
Q/2 + TTNP3 (3/12 – 11/12)	0.095	0.014	0.055	0.193	0.015	0.003	0.006	0.027	84.1

Afterwards, the CW was bioaugmented with 15 g of the bacteria (*Sphingomonas* sp. strain TTNP3) diluted to 20 L of tap water and added to the surface of the HSF constructed wetland. During this period the BPA removal was about 84%, which is not statistically different from the non-bioaugmented operation previously reported. However, further analysis is not included, since it was not between the objectives of this thesis.

Finally, it should be noted that the reduced removal efficiency during the last periods of operation could also be attributed to the healthy condition of the wetland plants, which suffered a period of drought when the wastewater was not continuously fed to the system.

A review on the literature of BPA removal using artificial wetlands with different plant species, showed similar levels of performance. BPA and nonylphenol (NP) were investigated in a pilot scale experiment conducted by Toro-Velez et al. (2016). The system consisted of three HSF-CWs in parallel, of 27 m^2 each and HRT of 1.8 d^{-1}. Removal efficiency of the 8.8 μg L^{-1} BPA concentration in the influent, was about 70-74 % in both pilots, planted with *Heliconia psitacorum* and *Phragmites australis*, whereas, non-planted CW achieved a removal of 62%. Avila et al. (Ávila et al., 2010) working in a two stages HSF-CW system planted with *Phragmites australis*, with a theoretical HRT of 3.5 days, reported removal efficiency of 85-99% (with nominal influent concentration 1 μg L^{-1}).

Removal mechanisms of organic contaminants in artificial wetlands, are affected by a variety of parameters, rendering these processes highly complex. A recent review (Garcia-Rodríguez et al., 2014) compiled removal mechanisms of EOCs occurring in biologically based treatment systems, which are primarily divided to biotic and abiotic mechanisms. Among the abiotic processes, sorption onto solid particles is presented as the prevailing mechanism for the removal of hydrophobic compounds such as BPA, hence it is presented as a plausible scenario in our study. It is worth noting that biodegradation of xenobiotics show antagonistic relation with sorption, since they become less bioavailable for degradation from microorganisms (Paranychianakis et al., 2006).

In horizontal subsurface flow systems, saturated substrate creates optimum conditions for anaerobic biodegradation that is the prevailing process in contrary to aerobic. Higher efficiency of aerobic pathways for the degradation of EOCs, is highlighted by several authors (Garcia-Rodríguez et al., 2014; Zhang et al., 2012; Hijosa-Valsero et al., 2010b). However, articles on biodegradation of xenobiotics under anoxic conditions, have revealed that this is not only an additional pathway, but occasionally the single one (Zhang and Bennett, 2005). Water depth is a parameter that affect the amount of oxygen, therefore the efficiency and the mechanisms involved. Highest efficiencies obtained in the shallowest layers, are attributed to the superior conditions for oxygen supply, as well as to the denser rooting system (Garcia-Rodríguez et al., 2014; Tore et al., 2012; Song et al., 2009).

In this study the shallow depth of the media of 45 cm (gravel), seems to be in favor of a sufficient BPA removal. Moreover, the presence of helophytes stimulates aerobic processes in the root zone, through the oxygen release from their roots (Stottmeister et al., 2003). What is more, root exudates (organic acids, sugars, amino acids etc.) can be used from the microorganisms as carbon and energy source, stimulating natural biodegradation (Truu et al., 2015). Stottmeister et al. (2003) published a well sited review in which the crucial role of microorganism in the transformation and mineralization of organic contaminants is being

highlighted. Based on the above knowledge, in our system, a high percentage of BPA removal could be attributed to microbial degradation.

Vegetation in CWs acts both as an oxygen supplier as well as a biosystem provider for the microorganisms, is being posed as an exceptional contributor for the remediation (Paranychianakis et al., 2006). Plant uptake and metabolism of hydrophobic organic compounds is presented as less important than microbial degradation, as afore-mentioned in this manuscript. However, uptake of BPA in plant tissues or metabolization into other compounds with less endocrine-disrupting activity, has been indicated in several research studies (Lu et al., 2015; Dodgen et al., 2013; Trueman and Erber, 2013) and could be suggested as a possible pathway in this experiment, even of lesser importance.

5.5.2. Wastewater quality parameters of the HSF-CW

In addition to BPA removal several other process parameters were measured; namely, Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD), pH, Electrical Conductivity (EC), Total Nitrogen (TN), Total Phosphorus (TP) and Total Boron (Bo).

COD & BOD

The two periods of operation are readily seen in Table 24. Initially at low values of inlet COD until November 2013 was followed by a period of a higher COD. Some data for BOD are not presented in the graph and they were not included in the removal rates shown in Table 24, as it was considered that were not reliable, due to analysis failure. BOD removal at the effluent measured higher values than 40 mg L^{-1}, thus over the limits for wastewater effluent (directive 91/271/EEC). COD removal was measured at 43.4 and 53.7%, corresponding to effluent values of 100.7 and 114.9 mg L^{-1} respectively. Therefore, even if the removal was not as high as expected, effluent concentrations were below the limits for WW effluents (125 mg L^{-1}) specified by the directive.

Tore et al. (2012) reported BOD removal efficiency in a full scale CW system of 60-85%, whereas, Chen et al. (2015) measured a reduction from 42.6 mg L^{-1} to 33.4 mg L^{-1}, using a 30 m^2 SSF-CW, as a part of an integrated CW system. Zhang, summarizing wastewater treatment in developing countries and subtropical regions using CWs (Zhang et al., 2015; Zhang et al., 2014b), demonstrated BOD removals of 53-90% and COD of 43-96%, in full-scale applications (Table 23). Vymazal (2010) also reported 75% BOD removal from HF-CWs, as a mean value of 367 CWs examined. Further concentrations in the influent and other characteristics of the systems (HLR, plant species and effluent values) are available in the referenced paper. Overall, a mean of 75% BOD removal is reported in this review. Finally, using a hybrid CW for the removal of EOCs, Avila et al. (2014) experienced an overall removal of 99% of the 320 mg L^{-}

[1] BOD in the influent. In the researche's study, individual removal of the HSF section was 36% of the 7 mg L^{-1} measured in the influent of this section, after passing from an imhoff tank and a vertical wetland. In terms of COD removal, the same authors reported removals of 36 and 34% respectively, from the initial influent concentrations of 405 mg L^{-1} and 29 mg L^{-1} for the two sections of the hybrid systems, respectively. Theoretical HRT within this bed was 2.3 d and covered an area of 229 m^2.

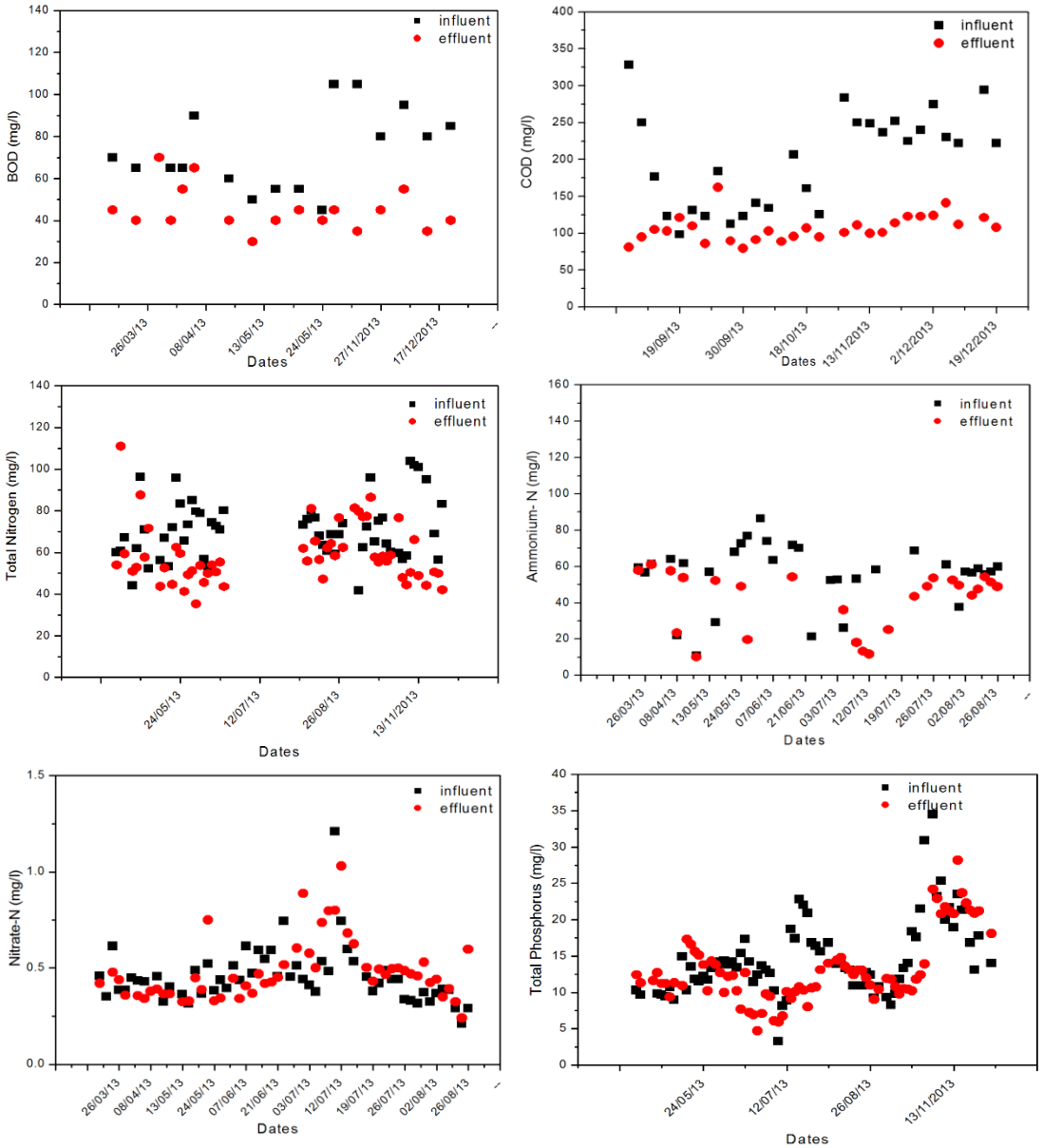


Figure 39. BOD, COD, total nitrogen, ammonium-nitrogen, nitrate-nitrogen and total phosphorus variation in the influent and the effluent, during the experimental period.

Although no specific analysis for the removal mechanisms was conducted in the present study, sulphate reduction, denitrification, anaerobic methanogenesis and aerobic respiration are stated as some of the major mechanisms of COD removal (Villaseñor Camacho et al., 2007). In the

review study of Zhang et al. (2015) COD removals after remediation in large scale HF-CWs, showed a variation from 43 to 96%, with an overall mean of 66%, for average effluent concentration of 65 mg L^{-1}. Specific characteristics of these data are presented anew in the referenced article.

Table 23. Comparative presentation regarding treatment efficiency of HSF-CW systems. Data collected by (Zhang et al., 2015).

Removal %	Type	Dimensions (m) (L x W x D)	HRT (days)	TSS	BOD_5	COD	NH_4-N	NO_3-N	TN	TP
Kenya	Municipal WW & secondary	7.5 x 3.0 x 0.6	-	72,3	60,7	42,8	26,4			42,9
	Municipal WW & secondary	7.5 x 3.0 x 0.6	-	72.9	53.0	44.0	17.1	22.0		57.1
India	Municipal sludge and tertiary	69 x 46 x 0.3	5.1	81	90	72	-	-	67	75
Costa Rica	Greywater & secondary	14 x 1.2 x 0.6	24	-	-	99.4	-	-	31.2	-
China	Municipal WW & secondary	33 x 3 x 0.5	3	-	90	70	50	-	46	60
Taiwan	Swine effluent secondary	9.5 x 2.6 x 0.7	8.5	96	91	84	22	54	24	47
China	Municipal WW & secondary	80 x 30 x 1.5 58 x 20 x 1.6	11.5 h 8 h	86.8	86.4	76.7	-	44.9	81.7	
El Salvador	Municipal WW & secondary	18.3 x 7.3 x 0.6	-	84.1	-	56.2	-	-	39.3	-
Vietnam	Municipal WW & secondary	12 x 1.6 x 1.1	-	82.6	78.3	70.6	62.6	42.7	50.0	69.7
Sri Lanka	Municipal WW & secondary	1 x 25 x 0.6	18	65.8	65.7	40.8	74.8	38.8	-	61.2

Nitrogen compounds

Total nitrogen includes organic nitrogen, nitrite, ammonium and nitrate. Removal of TN during the experimental period was approx. 19% of the 72.5 mg L^{-1} in the influent (Fig. 39). Effluent value of 59 mg L^{-1} exceeds the limits determined by the guideline 91/271/EEC, for sensitive areas (10 mg L^{-1}). In general, nitrogen removal in HF CWs is primarily attributed to nitrification/denitrification (Stottmeister et al., 2003), though, these systems are not effective in the removal of ammonia because of the low nitrification intensity caused by the absence of oxygen in the filtration bed (Vymazal, 2014). Different mechanisms are responsible for TN removal in CWs; absorption by plants (nitrate and ammonia), plant uptake, aerobic nitrification and anoxic denitrification, ammonification of organic nitrate and NH_3 volatilization (Villaseñor Camacho et al., 2007). What is more, experiments with planted and non-planted wetlands showed the contribution of plants to TN removal, that is depicted from the $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ balances (Villaseñor Camacho et al., 2007). As shown in Table 23, removal of TN from large

scale HF-CWs has been found to fluctuate between 41-96%, with an average value of 52% (Zhang et al., 2014b).

Table 24. Overall performance of the HSF-CW: mean, minimum and maximum values of the process parameters. S.E. indicates the standard error of the means.

Parameter	Influent				Effluent				Removal
	Mean	S.E.	Min	Max	Mean	S.E.	Min	Max	(%)
pH	7.58[a]	0.03	6.80	8.10	7.71[b]	0.03	7.10	8.60	-
EC (mS cm^{-1})	1.65[a]	0.05	0.96	2.51	1.69[a]	0.05	1.06	2.66	-
BOD (mg L^{-1}) (5/3-24/5)	74.1	3.9	45.0	90.0	46.3	3.6	30.0	70.0	37.4
BOD (mg L^{-1}) (13/11 – 20/12)	91.6	4.8	80.0	105.0	42.5	3.1	35.0	55.0	53.6
COD (mg L^{-1}) (10/9 – 29/10)	178.0	22.8	98.5	428.0	100.7	4.9	79.7	162.0	43.4
COD (mg L^{-1}) (5/11 – 20/12)	248.3	4.9	222.0	294.0	114.9	3.5	100.0	141.0	53.7
Total Nitrogen (mg L^{-1})	72.5[a]	2.4	41.9	156.0	58.9[b]	1.9	35.3	111.0	18.8
Ammonium-N (mg L^{-1})	55.7[a]	3.1	10.9	86.5	41.5[b]	3.3	10.1	61.1	25.5
Nitrate-N (mg L^{-1})	0.85[a]	0.39	0.21	22.8	0.70[a]	0.2	0.24	10.7	17.6
Total Phosphorus (mg L^{-1})	15.7[a]	1.1	3.3	87.3	13.9	1.1	4.7	85.5	11.2
Boron (mg L^{-1})	0.40[a]	0.03	0.21	0.66	0.26[b]	0.01	0.15	0.39	35.0

[a, b] mean values followed by a different symbol are significantly different ($p < 0.05$)

With respect to ammonium nitrogen, a mean dissipation of 25.5% of the initial 55.7 mg L^{-1} was observed, over the experimental period (Table 24, Fig. 41). The influent concentration of nitrate nitrogen (0.85 mg L^{-1}) was reduced by 17.6%, much lower than the effluent standards of 10.0 mg L^{-1}. NO_3-N was expected to be higher in HSF-CWs in contrary to VSSF wetlands, although the reverse has also been highlighted (Zhang et al., 2014b) . Removal of NH_4^+-N in HF-CWs is mainly attributed to plant uptake and nitrification, which is affected by environmental parameters (pH, temperature, dissolved oxygen) (Villaseñor Camacho et al., 2007; Li et al., 2014a). Findings noted in the bibliography indicate the higher removal of ammonium nitrogen in vertical flow systems, due to the higher oxygenation in the bed. Notwithstanding some studies were not in accordance with the aforementioned (Zhang et al., 2014b). Removal of ammonium nitrogen was inhibited (18.4%) in a non-planted CW in the absence of aeration, whereas, with the assistance of artificial aeration in a non-planted mesocosm, a removal of about 98% was achieved (Li et al., 2014a). Treatment efficiency of 30 and 39% for HSF-CWs were reported in a review study of Vymazal (Vymazal, 2010), when the effluent concentrations were 36 and 40 mg L^{-1} respectively. Zhang et al. (2014b) as previously described, regarding

large scale HSF systems, presented low removals of 17, 22 and 26% (effluent concentrations were 19, 1.4 and 19 mg L^{-1} respectively) although efficiency reached the highest value of 75% in a case of much lower effluent value of 4 mg L^{-1}.

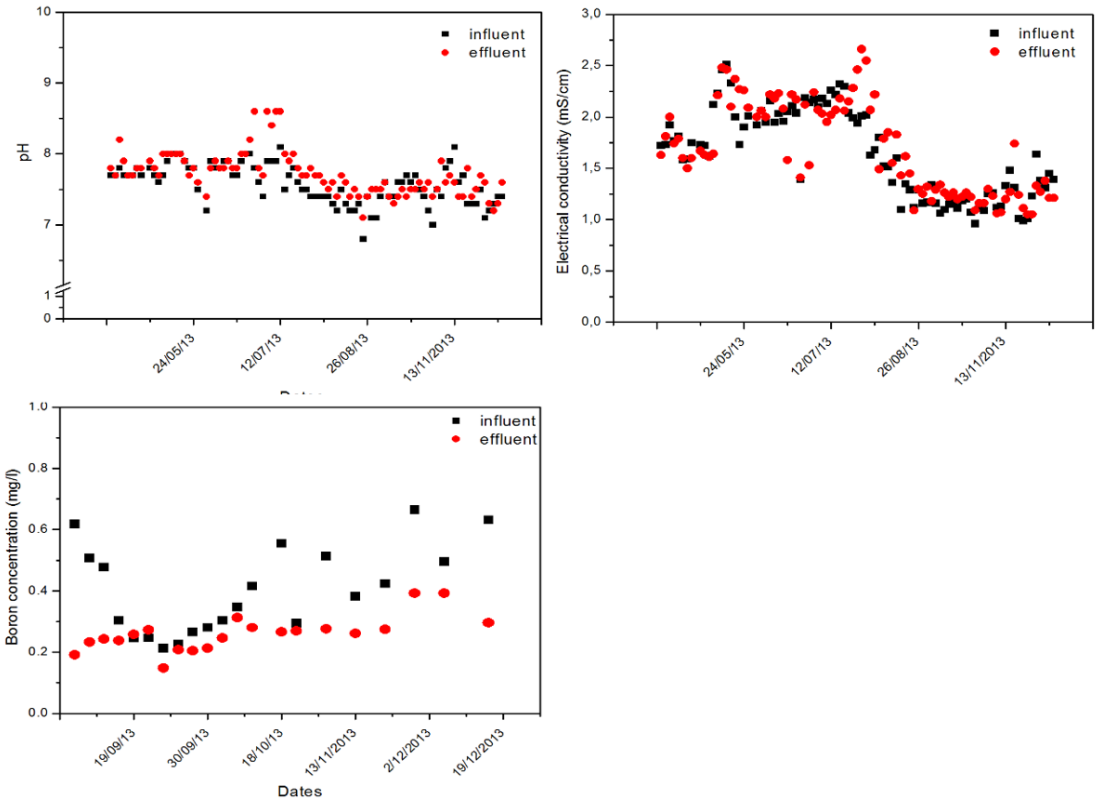


Figure 40. pH, electrical conductivity and boron concentration variations in the influent and the effluent, during the experimental period.

Total Phosphorus

In terms of total phosphorus, HSF exhibited low removal by 14% of the 13.7 mg L^{-1} influent concentration (Table 24, Fig. 39). Phosphorus can be in several forms in a CW; dissolved forms, dissolved associated with suspended solids, as a component of biomass or in the structure of soil particles, or sorbed on its particles (Fonder, 2010). Retention of P is considered to be relatively low in all types of artificial wetlands (Vymazal, 2010), since the major removal processes, sorption and precipitation, are not favored by subsurface flow systems, due to inert materials used in the substrate (Vymazal, 2010). In the latter study, efficiency of 50% is reported for a HSF-CW with influent concentration of 9.6 mg L^{-1}. With reference to the above mentioned review (Zhang et al., 2015) an average removal of about 70% (4.35 mg L^{-1}) of the phosphorus concentration is presented. Masi et al. (Masi et al., 2013) reported an average of 43% for TP, by a multi-stage CW system from urban wastewater. Tore et al. (Tore et al., 2012) reported a treatment efficiency of TP between 28 and 41% from urban wastewater.

pH- Electrical conductivity

The pH values did not change significantly from inlet to outlet simultaneously and over time. The mean value of pH was about 7.6, which is a typical value for CW (Table 24, Fig. 40). These findings are consistent with other researches, such as Zhang et al., (2010). With respect to EC, normally, in a classical wastewater treatment plant, EC values should decrease. However, in constructed wetlands the evapotranspiration may mask the removal of salts (Caselles-Osorio and Garcia, 2007). Indeed, no difference in the wastewater electrical conductivity between inlet and outlet was observed (Table 24, Fig. 40).

Boron

Boron compounds are used in glass industry, soaps, detergents and flame retardants, as more as in pharmaceuticals, pesticides and fertilizers (World Health Organisation, 2016). Irrigation with reclaimed water can reveal a significant toxicity problem for crops, in terms of boron and should not exceed 4 mg L^{-1} (Türker et al., 2014). Türker et al. (2014) compiled boron removal in CW systems, unraveling the related chemistry behind the elimination processes. Boron concentration in this study was reduced to values below 0.3 mg L^{-1} (Table 24, Fig. 40), less than the typical values for effluents that are around 0.75 mg L^{-1} (Rowe and Abdel-Magid, 1995).

5.5.3. Concluding remarks

The average removal efficiency for BPA was 98%, under nominal conditions in summer period. However there was significant sensitivity in the system to HRT changes, in conformity with the small-scale HSF-CW experiment of this study. Removal performance was decreased when HRT switched to half and started to increase again, after increasing HRT. Bioaugmentation with *Sphingomonas* sp. strain TTNP3 shown a very slight increase on the removal of BPA. Almost all other examined water quality parameters observed, were exhibited moderate low removal efficiencies. Mean COD concentration in the effluent was 101.3 mg L^{-1}, whereas, BOD was measured 46.4 mg L^{-1}. Average removal between 10-25% was observed for nitrogen and phosphorus.

CHAPTER 6. OVERALL CONCLUSIONS

Constructed wetlands are presented as a promising, low cost, socially-friendly technology which has been successfully applied worldwide during the last decades for wastewater remediation from nutrients, organic matter and selected organic pollutants, demonstrating a major advantage of CWs: the coexistence of different biodegradation pathways in a single treatment system (Hijosa-Valsero et al., 2010a). Research attempts on EOCs removal from municipal wastewater have witnessed substantial achievements throughout this period (Ahmed et al., 2016). However, research findings on specific groups of EOCs are yet to be fully understood. Among them are endocrine disrupting chemicals (EDCs) and antibiotics (Li et al., 2014b; Papaevangelou et al., 2016). As stated, current knowledge on removal efficiencies of CWs, the mechanisms involved and the impact of design and operational parameters is still limited. Moreover, rigorous experiments and the selection of the appropriate plant species for this purpose are of high importance. At the current stage of research, concentration on a specific plant is recommended in order to obtain more unambiguous results.

In this regard, several experiments were designed from small-scale to pilot-scale, treating contaminated soil, groundwater and real wastewater. To the extent of our knowledge, this is the first study that *J. acutus* has been investigated as a single vegetation species on soil and gravel based experiments for application in HSF-CWs for the treatment of real municipal wastewater, contaminated with BPA and antibiotics. Based on the findings of the present study and aforementioned discussion, the main conclusions can be summarized as follows:

- In pot experiments with soil substrate, planted treatments with *J. acutus* and *T. parviflora* showed higher BPA removal in comparison to non-planted ones. Degradation rate was found, in cases, to be two to three times higher by the presence of plants.

- *J. acutus* exhibited significant contribution on the removal of BPA, CIP and SMX in hydroponic cultures. The resultant removal capacity of BPA exceeded 96%, even when plants were exposed to a concentration of 50 mg L^{-1} of the compound and concentration of heavy metals reached the highest levels. *J. acutus* plants exhibited their potential to accumulate heavy metals in both root and leaf tissues in considerable amounts. The tolerance of the helophytic species to organic and inorganic stress was also demonstrated.

- Application of a rhizodegradation pilot demonstrated satisfactory results, regarding the removal of BPA from contaminated groundwater. It was shown to be reliable and robust within reasonable input oscillations of the BPA concentration and inlet volumetric flow rates.

- Performance of the HSF-CW for the removal of BPA from contaminated municipal wastewater was satisfactory and in conformity with other pilot studies. The ratio of the removed BPA mass over the mass entering the system was 46% higher in the presence of plants. The system demonstrated high sensitivity in terms of BPA removal with respect to changes in HRT. CIP exhibited moderate removal in the control wetland. SMX appeared to be recalcitrant to remediation with variable removals, indicating a complex behavior.

- In the larger scale HSF, average removal efficiency for BPA was 98%, under nominal conditions in the summer period. Significant sensitivity in the system with changes of HRT was also revealed.

Coexistence of different plant species and CW types (hybrid systems) have been reported to have a better performance because of the combined mechanisms involved. However, elucidating the performance of individual systems and plant species is of high importance for the better understanding of the processes involved. This insight should lead to a more efficient design of CWs. In order of a plant species to be suitable for CW applications, several criteria should be met. For instance, tolerance to contaminated wastewater, high biomass production and deep, extensive root system to support rhizosphere processes. In this study, *Juncus acutus* was considered to meet these requirements: exhibited fast growth rate and intense rhizome propagation, whereas, its leaves remained erected and green throughout the experimental period. Overall, *J. acutus* plants demonstrated efficient performance as a single plant species in a HSF-CW, enhancing the capacity of the system to treat organic pollutants for municipal wastewater and represent a promising species worth to be further investigated.

Future perspectives and recommendations

There are still many unanswered questions in CWs and future research could focus on:

- The mechanism of BPA mineralization within the plant.
- Elucidation of degradation pathways and improvements in analytical methods for pharmaceuticals.
- Long term effects of heavy metals pollution, in order to assess the accumulation potential of plants.

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1. **Christofilopoulos, S**., Syranidou, E., Gkavrou, G., Manousaki, E., Kalogerakis, N., 2016. The role of halophyte *Juncus acutus* L. in the remediation of mixed contamination in a hydroponic greenhouse experiment. *J. Chem. Technol. Biotechnol*. 91, 1665–1674.

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* Both researchers contributed equally to this manuscript.

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1. **Christofilopoulos**, **S**., Kaliakatsos, A., Triantafyllou, K., Voutsadaki, S., Nikolaidis, N., Venieri, D., and Kalogerakis, N. Evaluation of a pilot scale constructed wetland for municipal wastewater treatment, with emphasis to EOCs and pathogens removal. *Water Res*., (in preparation).

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1. Kalogerakis, N., **Christofilopoulos, S.** Rhizodegradation in constructed wetlands. In: Hochstrat R, Wintgens T, Corvini P, editors. Immobilized Biocatalysts for Bioremediation of Groundwater and Wastewater. London: IWA Publishing; 2015. p. 97–105.

CONFERENCE PRESENTATIONS

1. **Christofilopoulos**, S., Kaliakatsos, A., Triantafyllou, K., Voutsadaki, S., Nikolaidis N., Danae, V., Kalogerakis, N. 2017. Evaluation of a constructed wetland for wastewater treatment, with emphasis on the removal of emerging organic contaminants and antibiotic resistant bacteria. *International Conference on Environmental Engineering and Management*, Bologna, Italy (oral presentation).

2. **Christofilopoulos, S**., Syranidou, E., Gkavrou, G., Manousaki, E., Kalogerakis, N., 2015. The role of halophyte *Juncus acutus* L. in the remediation of mixed contamination in a hydroponic greenhouse experiment. *6th European Bioremediation Conference*, Chania, Crete (oral presentation).

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