

Extended Abstract of Ph. D.

The main goals of this study were to i) assess the performance of full-scale secondary biological treatment technologies (i.e., conventionally activated sludge (CAS) and membrane bioreactor (MBR)) in relation to the removal of chemical contaminants of emerging concern (CECs) and microbiological contaminants, ii) investigate the potential uptake and proliferation of these contaminants into crops irrigated with reclaimed water in real-field conditions, iii) evaluate the efficiency of ozonation process operated in batch and continuous mode, in simultaneously removing the inherent chemical CECs and microbiological contaminants, from CAS or MBR treated wastewater, and also the toxicity (i.e., phytotoxicity and ecotoxicity) of the effluent after ozonation, and iv) perform a comprehensive techno-economic evaluation of continuous-mode ozonation process based on the achieved removal efficiencies of the examined contaminants.

The investigation of the inflow and outflow of the actual treatment plants applying CAS and MBR treatment technologies revealed their inability to entirely remove and inactivate the examined chemical CECs and microbiological contaminants. Regarding the removal of the chemical CECs, several factors such as the applied treatment technology, along with their operating conditions (i.e., hydraulic retention time (HRT), solid retention time (SRT), mixed liquor suspended solids (MLSS)), the plant configuration, the physicochemical parameters of the examined compounds, and the different environmental factors (i.e., temperature) seemed to affect the presence of these compounds in the final effluents. Regarding the inactivation and the removal of selected antibiotic-resistance determinants (antibiotic-resistant bacteria (ARB) & antibiotic resistance genes (ARGs)), the two treatment plants showed that both were capable of reducing the examined contaminants to a lower abundance but were not able to remove them entirely. The results concerning the inactivation and removal, were approximately the same, indicating this way that despite the operation of chlorination after the CAS treatment technology, the MBR treatment can indeed provide similar results since it rejects the bacteria and sludge flocs including antibiotic resistance determinants with molecular weight greater than the membrane pore size. However, the inability of both treatment technologies to entirely remove and inactivate the microbiological contaminants create the need to add additional steps of treatment capable of bringing the examined contaminants to concentrations lower than the limit of detection (LOD). Whilst reclaimed water reuse for irrigation is recognized as one of the indispensable practices for the mitigation of water scarcity, challenges such as the presence of chemical CECs and microbiological contaminants act as a limiting factor in effective reuse practices. These results

led to the conclusion that further studies should be conducted to identify the appropriate processes to be applied for the optimization of the removal of the examined chemical CECs and microbiological contaminants in order to minimize as much as possible the threat of spreading the CECs and antimicrobial resistance (AMR) in the environment through reusing the reclaimed water.

The investigation of the lettuce cultivation experiment in lysimeters under real-field conditions, irrigated with reclaimed water after CAS and MBR treatment, revealed that the occurrence, accumulation (i.e., in soil and leachate), and uptake (i.e., by crops) of the examined chemical CECs is at some degree, inevitable. The results revealed that at the initiation of the experiment (Day 0) the examined pharmaceutical compounds were all present in both the CAS- and the MBR-treated effluents. For the CAS-treated effluents erythromycin (ERY) with 63.4 ng L^{-1} has the highest concentration, while carbamazepine (CBZ) with 81.3 ng L^{-1} had the highest concentration in the MBR-treated effluents. The results indicated that storage of the reclaimed water for 40 days in the storage tanks under the harsh environmental conditions prevailing during the experimental period had affected the concentration of the examined compounds. Regarding the soil irrigated with CAS-treated effluent, CBZ had the highest concentration ($23.5 \text{ } \mu\text{g kg}^{-1}$) among the detected and quantified compounds in the top-soil layer followed by azithromycin (AZM), clarithromycin (CLA) and ERY in descending order, in terms of their concentrations. Similar results were obtained with the MBR-treated effluent. More precisely, AZM and CBZ with concentrations of 1.00 and $0.99 \text{ } \mu\text{g kg}^{-1}$, respectively, were quantified in the top-soil layer, while they were also detected in the soil layer of 20-40 cm, where CLA was detected however below the method of quantification (MQL). The detection and quantification of the examined compounds in the different soil layers of the lysimeters' soil horizon showed that once these compounds introduced to the soil via reclaimed water irrigation, undergo changes through several chemical, physical and biological processes (biotic and abiotic) influencing this way the mechanisms of sorption in the soil compartment. The concentration of the detected compounds decreased with increasing soil depth indicating this way that the fate and concentration of these compounds in soil, is closely related to their physicochemical properties and the soil characteristics. Moreover, soil is a living, dynamic system that greatly influences the fate and bioaccessibility of organic micropollutants present in this compartment. Another interesting finding obtained from the results of the lysimeter experiments is that the presence or absence of crop plants from the lysimeters affected the detection and quantification of the examined compounds. Fewer compounds were detected and lower concentrations were quantified in the lysimeters without lettuces. Regarding

the presence of the examined pharmaceuticals in lettuces irrigated with CAS-treated effluent it was shown that CBZ had the highest concentration with $30.9 \mu\text{g kg}^{-1}$ followed by diclofenac (DCF) with $7.18 \mu\text{g kg}^{-1}$, while the rest of the examined compounds although detected, their concentrations were slightly below the MDL. However, in lettuce samples from the treatment line irrigated with MBR-treated effluents only CBZ was detected with concentrations below the MQL. These results may be attributed not only to factors related to the physicochemical characteristics of the examined pharmaceutical compounds (i.e., hydrophobicity, solubility and ionic form of the compounds), but also to physiological plant variables (e.g., plant lipid content, detoxification and metabolism system, growth and transpiration rates) affecting the uptake potential and differential translocation factors. The structure of the lysimeter experimental setup provided the ability to investigate the potential leaching of the examined pharmaceutical compounds from soil. The results revealed that only CBZ was leached out from the lysimeters, with either or without plants, when CAS-treated effluents were used. The detection and quantification of CBZ in leachate samples demonstrated that the potential contamination of groundwater with CECs through reclaimed water irrigation should not be ignored. It is known that pharmaceutical compounds are subject to transport and biodegradation when they enter soil. The results revealed that CBZ has lower sorption affinity and higher mobility in soils, and therefore could be easily transported from soil surfaces to the aquifer. The persistence and translocation of CBZ in the soil horizon is due to its increased half-life and its neutral character can justify the persistence of CBZ in soil and its migration into lower soil depths ending thereafter in ground water.

The investigation of the bacteria community structure through the 16S rRNA amplicon sequence was performed in the samples of reclaimed water, soil, lettuce, and leachate. Overall, only 25.6% of the samples managed to pass the quality control, with the majority of the sequence being rejected due to chimeric amplicons and chloroplasts, especially the epiphytic samples (lettuces). Moreover, some of the leachate samples failed to pass the quality control due to the low quantity of the extracted DNA, therefore these samples were excluded from the analysis. The ASVs data were analyzed in terms of their α - (within-sample) and β - (between sample, compositional differentiation) diversity along with the differential abundance between the treatment of the participating ASVs. The results from the α -diversity revealed that the microbial community structure in the TW and MBR-RW samples seem to increase during the storage in the tanks for 40 Days, while the opposite was observed for the CAS-RW. This might be attributed to the residual chlorine present in the CAS-RW since this particular reclaimed water (RW) comes from full-scale UWTPs that utilize CAS treatment technology followed by sand filtration and

chlorination. On the other hand, the increased trend of the microbial diversity that was observed in the MBR-RW might be attributed to the activities of the microbial groups that already exist and managed to pass the membrane filtration barrier. Regarding the soil samples, the microbial diversity of the soil irrigated with TW and CAS-RW seemed to differentiate with increasing soil depth and higher diversity was observed in the top-soil layer (0-20 cm), while a lower diversity was observed in the middle (20-40 cm) and deepest soil depth (40-60cm). These observations might be attributed to different parameters such as the environmental conditions and the soil moisture which can influence the composition of the soil bacterial community. The analysis in terms of the β -diversity, shows clear differences between the fresh and stored CAS- and MBR-RWs samples indicating this way the evolution of the microbial community in terms of amplicon sequence variants (ASVs)/taxa abundance in these samples, while no differentiation was observed in the case of the TW samples. However, the β -diversity analysis showed that the microbial community of the soil samples were compositionally similar in the different irrigation regimes. Differential abundance tests were carried out to investigate the differential abundant putative pathogens between the examined samples from the different irrigation treatment lines. In the tube well water-irrigated samples, only the phylum of *Proteobacteria* (*Gammaproteobacteria*) were detected, which is consistent with the scientific literature reporting the detection of this phylum even in fresh water. In the CAS-RW samples a higher number of putative pathogenic taxa was detected (42 different strains) belonging to *Proteobacteria* (N=28, 66.6%), *Firmicutes* (N=10, 23.8%) and *Actinobacteria* (N=4, 9.5%). The majority of the detected putative pathogenic strains (24 out of the 42 (57.1%)) were spotted in the soil samples precisely in the top-soil layer indicating that with increasing soil depth the microbial community and structure changed. This might be attributed to environmental conditions such as aeration and organic substrate supply which decreased with the increasing soil depth, leading to reduction of the bacterial species number and the elimination of bacteria unable to withstand harsher conditions. In total, 29 strains were detected in the MBR-RW irrigated samples with most of them belonging to *Proteobacteria* (N=20, 68.9%) followed by *Actinobacteria* (N=9, 31.0%). The phylum of *Actinobacteria* showed higher differential abundance in the samples of RW (Day 0 and 40) in comparison with that in the CAS-RW. This might be associated with the size of some of these bacteria, being smaller than the pore size of normal or aged membranes at certain growth stages and in environmental oligotrophic settings. In general, the result of the 16S rRNA amplicon revealed that the storage of the reclaimed water influenced the structure of the microbial community. As expected, the soil microbial community was influenced by the reclaimed water irrigation in terms of the ASV analysis and especially the top-soil layer and where the results also

revealed that with increasing soil depth lower influence existed for the reclaimed water irrigation. Moreover, the results regarding the differential abundant putative pathogens did not provide any clear indication for their accumulation and proliferation in the different environmental compartments irrigated with reclaimed water. However, it is recommended that further investigation is performed in order to understand in more depth the phenomena taking place during the irrigation with reclaimed water.

The ozonation process operated in batch mode achieved a complete removal of the examined antibiotic compounds. More precisely, the findings in both wastewater matrices examined (CAS- and MBR-treated effluents) revealed that the contact time of 5 min with ozone doses of $1.5 \text{ gO}_3 \text{ gDOC}^{-1}$ and $1.0 \text{ gO}_3 \text{ gDOC}^{-1}$ were adequate to completely degrade the parent compound of the examined antibiotics, in CAS- and MBR-treated effluents, respectively. It was also shown that the inactivation of the examined bacterial species by ozone was governed by the CT. The model predicted CT requirements showed that to achieve a log reduction higher than 1, a higher CT is required for CAS- than MBR-treated effluents, indicating this way that the different quality characteristics of the wastewater matrix might influence the CT requirement for the inactivation of the examined microbial group. The ozone was effective in inactivating bacterial species carrying resistance to TMP, SMX, ERY and OFL. The abundance of most of the examined genes, including ARGs, was reduced to values below the LOD after ozonation for both wastewater matrices. Post-treatment storage for 72 h resulted in no regrowth of bacteria, while an increase in the 16S rRNA abundance was observed, indicating that non-investigated bacteria either survived ozonation or repaired, and hence might exist in the ozone-treated samples. No substantial mineralization (i.e., DOC removal) was achieved. Phyto- and eco-toxicity increased after the ozonation treatment for both wastewater matrices, indicating that the interaction of ozone and dE_fOM may result in the generation of potentially toxic oxidation products. Higher toxicity for CAS-treated effluents compared to MBR-treated effluents was observed after ozonation.

The findings of the continuous-mode ozonation demonstrated that it is a promising treatment technology for the elimination of both the selected antibiotics and antibiotic resistance determinants. The results revealed that low ozone doses accompanied by prolonged treatment time was needed to eliminate the parent compounds of the examined antibiotics at concentrations below their LOD in the spiked experiments, whereas the mildest conditions (i.e., lower HRT of 10 min for the same ozone dose of $0.125 \text{ gO}_3 \text{ gDOC}^{-1}$) were sufficient to degrade

the parent compounds in the non-spiked experiments. No substantial mineralization was observed at the end of the treatment time at all experimental conditions examined, a fact that may explain the enhanced phytotoxicity of the ozonated samples towards the tested plant species. This might be attributed to the formation of recalcitrant organic transformation products deriving from the oxidation of dE₇OM originally present in wastewater. Ozonation operated at high HRT and ozone doses (HRT 40 min; 0.25 gO₃ gDOC⁻¹) was capable of inactivating total as well as TMP- or SMX-resistant *E. coli*, with the simultaneous reduction of the abundance of the examined genes. However, the increase of the abundance of the 16S rRNA (which is a biomarker of the load of total bacteria) demonstrated that bacteria other than *E. coli* might still be present in the ozonated samples.

Overall, it was shown that ozonation upon its proper optimisation constitutes a satisfactory efficient treatment option for the removal of antibiotic-related microcontaminants from reclaimed water. The performance of the techno-economic evaluation of ozonation process showed that the low capital and operational cost of the ozonation process made it a well-fit process for tertiary treatment in a full-scale treatment plant. Nevertheless, it is suggested that after ozonation, post-treatment is required to overcome the main limitations of the process in relation to its toxicity production and the potential bacterial regrowth, in order to minimise the potential risks imposed on the receiving environments. Furthermore, in the light of the newly approved EU Regulation on the specific qualitative criteria regarding the reclaimed water reuse along with the risk management, it is becoming obvious that more holistic studies are required to contribute towards the improvement of the characteristics of the ozone-treated wastewater, while minimizing the possible risks related to the proliferation of antibiotic resistance determinants, potential bacterial regrowth and toxicity in environmental compartments. Having this in mind, the results of this study may be of high environmental significance in the framework of sustainable wastewater management.