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Partially saturated vertical surface flow constructed wetland for emerging contaminants and antibiotic resistance genes removal from wastewater: The effect of bioaugmentation with *Trichoderma*.

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## Abstract

The scientific significance of this work involved in investigating the removal of contaminants of emerging concern (CECs) and antibiotic resistance genes (ARGs) at pilot-scale with partially saturated vertical flow constructed wetland either bioaugmented or not with the *Trichoderma asperellum* (strain T34) by means of the non-target liquid chromatography high-resolution mass spectrometry and SmartChip™ Real-Time PCR methodological approaches. Bioaugmentation testing was successful as *Trichoderma* was able to grow in competitive conditions resulting from the use of secondary treated domestic wastewater. There was a clear relationship between the removal efficiency (RE) and the biodegradability as well as the sorption capacity of the identified compounds. The most easily eliminated compounds were those which are known to be readily biodegradable (e.g., nicotine). Interestingly, from 22 compounds with RE > 65%, 17 compounds have a log Dow between

0 and 3 (optimal plant uptake). The most significant beneficial effect was observed with benzotriazole and diclofenac. The most difficult compounds to be eliminated were the most polar ones ( $\log \text{Dow} < 0$ ) such as melamine. High variability in removal efficiency over the course of a four-week experiment remained the chief constraint for the proper assessment of treatment performances. Transformation products (TPs) were also investigated. N-oxide TPs and 14-hydroxyclearithromycin were formed in wetland treatment while TPs which were prone to undergo further N-dealkylation or hydroxylation reactions were partly eliminated. Bioaugmented wetland treatments caused a shift in the composition of ARGs but there was no statistical difference in average rates of ARGs removal.

#### Graphical abstract



**Keywords:** Antibiotic resistant genes; bioaugmentation; constructed wetlands; contaminants of emerging concern; transformation products; *Trichoderma*.

## 1. Introduction

Constructed wetlands (CW) offer robust and cost-effective treatment of domestic wastewaters in regions of low population density (e.g., small cities in rural areas) while meeting secondary treatment standards concerning the removal of conventional pollutants (e.g., bacteria, carbon and nutrients such as nitrogen and phosphorus, Varma et al., 2021; Rahim et al., 2022). In an attempt to reduce ground footprint and to improve the removal of total nitrogen (TN) and contaminants of emerging concern (CECs) including antibiotic resistance genes (ARGs), intensified systems such as aerated CW either with continuous or intermittent active aeration have been successfully implemented at the expense of higher capital and operational costs (Kahl et al., 2017; Avila et al., 2021a).

A combination of both low CBOD<sub>5</sub> and high oxygen availability is crucial for the transformation of the CECs with the lowest biodegradability (e.g., benzotriazole and diclofenac) for which a removal efficiency (RE) > 80% was only obtained during a period of highest performances (warm season, Chen et al., 2019). In such systems, oxidative processes enhanced the removal of compounds with high biodegradation potential, whereas non-biodegradable compounds were mainly removed by sorption to solid particles. However, more efforts are needed in the characterization of transformation products (TPs) to achieve a good process understanding at the microbial community level as well as the biochemical degradation pathways (Carvalho, 2020).

The so-called partial saturated vertical subsurface flow CW (VSSF-CW) has also shown enhanced removal of TN and selected CECs without the requirement of external energy input (Avila et al., 2021b). This result was mainly assigned to a more diverse and metabolically versatile bacterial communities including both the growth of aerobic and anaerobic bacteria due to the generation of redox gradients throughout the filter bed which establishes nitrification and denitrification conditions (Avila et al., 2021b). However, both saturated and anaerobic conditions, despite favoring

the elimination of certain antibiotics, may result in the spread of ARGs in bacterial communities (Aydin et al., 2015). VSSF-CW could remove ARGs from wastewater by directly eliminating microorganisms but different ARGs had different removal efficiencies implying the need of a screening of a large number of ARGs for the evaluation of water treatment performances. Overall, these results indicated that the studied wetlands were not able to significantly reduce either the bacterial load of the influent wastewater or the prevalence of most ARGs within the bacterial community (Monsalves et al., 2022).

Bioaugmentation appears to be a promising approach to improve the functioning of wetland treatments, considering the detection of natural mycorrhization in wetlands and that bacteria are the main driver of contaminant degradation processes (Tondera et al., 2021). At the pilot-scale, bioaugmentation has been successfully implemented to increase nitrification (Austin et al., 2019) by using commercially available liquid suspensions of nitrifying bacteria as well as denitrification in saline water by adding a salt-tolerant microbial inoculum (Wang et al., 2020). Inoculation with symbiotic arbuscular mycorrhizal fungi also tended to show a positive effect on the growth of some wetland plants (e.g., *Phragmites australis*) in phytoremediation experiments (Calheiros et al., 2019). However, little evidence exists to show that bioaugmentation of CW through bacterial or fungal inoculation substantially improves their efficiency in CECs and ARGs removal.

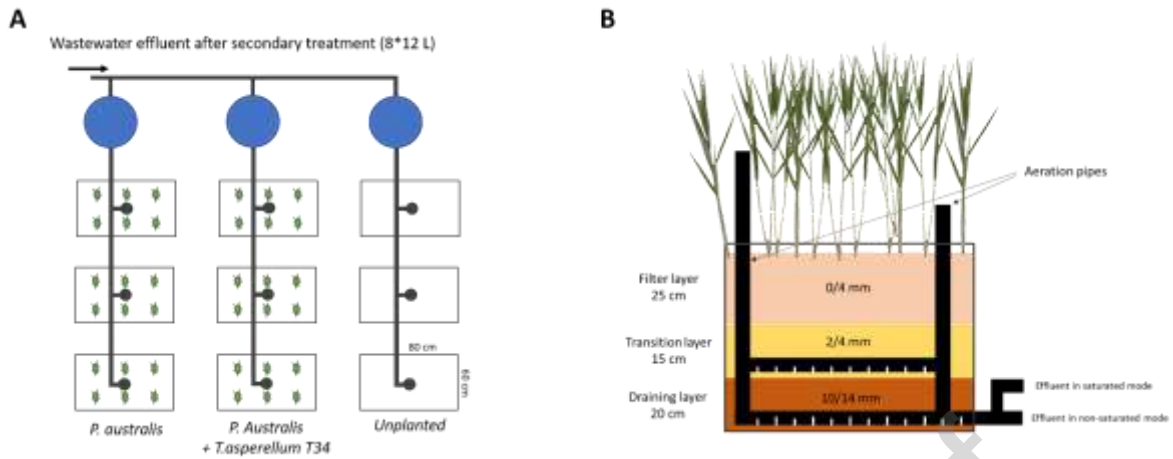
In general, vertical flow CWs have shown to be more effective in removing CECs from domestic wastewater compared to horizontal configurations (Avila et al., 2014; Carvalho, 2020) and saturated conditions seem to improve the removal of certain antibiotics like ciprofloxacin or ofloxacin and azithromycin (Avila et al., 2021b). In this context, we decided to inoculate at a pilot scale VSSF-CW with *Trichoderma* as (i) they are known to be plant symbionts which might promote plant performances and contaminants plant uptake (Guzman-Guzman, 2019), (ii) they possess a machinery of unspecific enzymes (e.g., laccase and peroxidase enzymes), similarly to *Trametes Versicolor*, which

makes their application in CECs removal attractive (Manasfi et al., 2020) and (iii) they are authorized to be used as biocontrol agents against native pathogens and can be released in the soil in case of wastewater reuse for crop irrigation without the need for additional treatment for their removal. Specific objectives included studies on i) the reduction of conventional water quality parameters (e.g., nutrients, carbon and bacteria), ii) the removal of a large set of CECs by non-target liquid chromatography-high resolution mass spectrometry (LC-HRMS) screening and iii) the removal of selected ARGs by means of a SmartChip™ Real-Time PCR methodological approach.

## 2. Material and Methods.

### 2.1 Description of the experimental set-up.

The present study was carried out with VSSF-CW units located outdoors at the facilities of the experimental platform for wastewater reuse in irrigation in Murviel-Lès-Montpellier (Southern France). The system was operated as tertiary treatment and was fed with real secondary treated domestic wastewater obtained by an aerated CW with a nominal treatment capacity of 3000 Inhabitant Equivalent and with additional treatment for phosphorus removal by precipitation with ferric chloride. The treatment system consisted of three lines of three CW units each and operated in parallel. Each unit was designed to treat 96 L/d distributed in 8 batches along the day and was partially saturated with water (0.2 out of 0.6 m height), allowing for a combination of oxic and anoxic conditions (see Scheme 1).



Scheme 1: Experimental design for studies on CECs removal in bioaugmented constructed wetlands.

The pilot consisted in three lines of three VSSF units (A) fed in batch mode with wastewater from secondary treatment. Each wetland unit had a surface area of 0.48, and a filter media depth of 0.6 m, consisting of a 0.25 m filter sand layer on top, a 0.15 m transition layer of fine gravel and a 0.20 m draining layer of gravel at the bottom (B).

The first line consisted of substrate and plants, while in the second line, *Trichoderma* was inoculated. A third line without plants was also implemented as a reference system for which only conventional water quality parameters were determined to check for the well-functioning of planted CW units. The beds were constructed inside polyethylene tanks. Each wetland unit had a surface area of 0.48 m<sup>2</sup> (0.8 m W × 0.6 m L), and a filter media depth of 0.6 m, consisting of a 0.25 m filter sand layer ( $\phi = 0/4$ , d<sub>10</sub> = 310  $\mu$ m and d<sub>60</sub> = 650  $\mu$ m) on top, a 0.15 m transition layer of fine gravel ( $\phi = 2/4$  mm) underneath, and a 0.20 m draining layer of gravel at the bottom ( $\phi = 10/14$  mm). Each unit was designed with a passive aeration system at the transition layer and a draining pipe at the bottom collecting water by gravity and driving the filtered effluent to the collector pipe. A polyethylene pipe distributed the pumped water uniformly 0.1 m above the bed surface. The planted macrophytes in wetlands were *Phragmites australis*. Wetlands were intermittently and simultaneously fed (one batch every two hours from 8 am to 10 pm). The average organic loading rate (OLR) and hydraulic loading rate (HLR) applied to each CW unit during the study were 10 g COD m<sup>-2</sup>/d and 200 mm/d,



respectively. CW units have been in operation alternating cycles of feed and rest since May 2021 so that the filter media and rhizosphere were well established and colonized by microorganisms. Bioaugmentation with *Trichoderma*. was conducted in June and July 2021 and sampling started 8 weeks after the last inoculation. The growth of *Trichoderma* was followed before and during the sampling campaign (see below for methodology). Influent and effluent samples (1 L) were taken as grab samples in amber, sterile glass bottles once a week for four consecutive weeks in September 2021. Three replicates were achieved in order to calculate standard deviation values of CECs concentrations as well as those of conventional water quality parameters concentrations (see below). For the analysis of ARGs, water samples (250 mL) were filtered through 0.2 µm pore-size membranes (0.2 µm polyethersulfone 47 mm diameter, PALL). Filters were then carefully folded and introduced separately into 2 mL sterile Eppendorf tubes and safely stored at -20 °C until DNA extraction. Sample aliquots (100 mL) were then taken for the analysis of conventional water quality parameters. Finally, for the determination of chemical contaminants sample aliquots (250 mL) were filtered through 0.45 µm cellulose filters and preconcentrated by solid phase extraction (SPE) also on the same day; SPE cartridges were frozen at -18 °C until analysis.

## 2.2 Chemicals and reagent

Analytical standards of benzotriazole, carbamazepine, diclofenac, propranolol, sulfamethoxazole, tramadol, venlafaxine, fluconazole, and their deuterated labelled compounds were purchased from Sigma-Aldrich, with a purity of greater than 95%. After preparation, the standards were stored in methanol at -18 °C. HPLC-grade methanol and acetonitrile were purchased from Carlo Erba. Nitrogen used for drying was purchased from Air Liquide. A Millipore Milli-Q Simplicity® UV system was used to obtain HPLC-grade water. Additional dilutions were also prepared in a methanol / water mixture (10:90, v/v). Oasis HBL SPE cartridges (200 mg / 6cc) were purchased from Waters Corporation, whereas PTFE 0.2 µm filters were obtained from Chromafil (Chromafil® Xtra CA 20/13). *Trichoderma asperellum* (strain T34) was a gift from the Department of Plant Biology, Faculty of Biology, University of Barcelona (Spain).

### 2.3 Analysis of conventional water quality parameters

For the measurement of the water quality parameters the Spectroquant® NOVA 60A was used, using a specific test kit for each parameter: NH<sub>4</sub>-N (Kit 114752), NO<sub>3</sub>-N (Kit 114543), NO<sub>2</sub>-N (Kit 114776), total nitrogen (TN, Kit 114763), PO<sub>4</sub>-P (Kit 114543), chemical oxygen demand (COD) (Kit 114540), and SO<sub>4</sub><sup>2-</sup> (Kit 114548) which were purchased from Sigma-Aldrich, France. Dissolved oxygen (DO), electric conductivity (EC) and pH were measured using portable probe WTW™ MultiLine™ 3420 Portable Digital Multiparameter (Fisher Scientific, France).

### 2.4 Analysis of CECs in water

Sample preparation, chromatographic separation, and mass spectrometry conditions are described in detail elsewhere (Tadić et al., 2022). Briefly, a generic approach was applied that consisted of SPE using OASIS HLB cartridges (200 mg sorbent, 6 cc). Extracts were analyzed on an HPLC Accelera 600 pump coupled to a Q-Orbitrap HRMS mass spectrometer (Thermo Fischer Scientific, Les Ullis, France) equipped with a heated electrospray ionization probe (HESI) source for detection. Chromatographic separation was conducted using a Waters XBridge BEH C18 (2.1 × 150 mm and 2.5 μm particle size) analytical column equipped with a pre-column. The chromatography assays involved a 10 μL injection volume, a 0.30 mL/min flow rate, and a binary gradient of water (A) and acetonitrile (B), both containing 0.1% formic acid. Full scan data were acquired at a resolution of 70,000 full width at half maximum (FWHM) with automatic gain control (AGC) of 10<sup>6</sup>, 250 ms of the maximum ion injection time, and scan range 100–1000 *m/z*. MS<sup>2</sup> and data-independent acquisitions (DIA) were achieved at a resolution of 17,500, two absolute collision energies (20 eV and 40 eV) isolation window 1 *m/z*, AGC of 5 × 10<sup>4</sup>, 150 ms of the maximum ion injection time, and a scan range of 50 - 1000 *m/z*. The non-target screening was performed with a home-made tool (Lestremau et al., 2023). The tool was developed using a machine learning approach aiming at improving the ranking of compounds identification. Raw data were converted in mzML format using MS convert software (Proteowizard, Adusumilli and Mallick, 2017) and then processed with the tool. Three types of databases, containing mainly environmental contaminants, were screened: a homemade database from the injection of

207 standards in positive mode (POS), a database provided by the vendor Agilent, which included 919 substances in POS, an extract of MassBank Europe database - Release version 2021.03 (<https://massbank.eu/MassBank/> (accessed March 04, 2022)). Only spectra containing high-resolution MS/MS information were considered representing a total of 1717 substances in positive ionization mode. Compound identification and ranking were then proposed by the tool. All compounds which ranked with a threshold score  $\geq 0.85$  (out of 1) were selected. From previous experience, this threshold represented a limit to avoid false positives, and compounds falling in this category could be classified as level 2 of identification confidence following the Schymanski scale (Schymanski et al., 2014). All the selected compounds were also manually reviewed to validate their identification, with experimental mass spectra compared to reference spectra and comparison of isotopic profiles. Water samples, fortified with the mixture of analytical standards at 10 ng/mL prior to extraction, served as positive control samples and as confirmation for the suspected screening. Furthermore, no difference in matrix effects between the inlet and outlet was observed; therefore, comparison of the peak areas was used to calculate the removal efficiency (RE). RE was calculated according to the following formula:

$$RE (\%) = 100 * \frac{\text{Peak area influent} - \text{Peak area effluent}}{\text{Peak area influent}}$$

## 2.5 Quantification and analysis of antibiotic resistance genes

Environmental DNA was extracted directly from the filters using the DNeasy PowerWater Kit (Qiagen, Venlo, Netherlands) according to instructions. Gene detection and quantification were performed using the SmartChip™ Real-Time PCR system (TakaraBio, CA, USA) by Resistomap Oy (Helsinki, Finland). A total of 72 primer sets were used to assay the genes of interest (list of all selected genes – Supporting material). Details are given elsewhere (Muziasari et al., 2016; Muurinen et al., 2017; Muziasari et al., 2017). The analysis of ARGs abundances and profiles was performed with R (version 4.2.0). ARGs abundances were normalized by 16s rRNA abundances. Principal component analysis

(PCA) was performed on the  $\log_{10}$  transformed data. The ARGs  $\alpha$ -diversity was calculated with Shannon and Simpson diversity indexes (Shannon and Weaver, 1949). The distribution of ARGs was done with the package *rstatix* (Kassambara, 2021) using Wilcoxon tests, followed by Benjamini-Hochberg test for the false discovery rate for multiple comparisons ( $p \leq 0.05$ ). The ARGs  $\beta$ -diversity was analyzed with Bray-Curtis (1957) and Jaccard dissimilarities (Jaccard, 1900). Firstly, the homogeneity of groups dispersions (betadispersion) was calculated. The effects of the different treatments were tested with the *adonis* PERMANOVA. The variation between samples was visualized by a Non-metric Multidimensional Scaling (NMDS) using the Jaccard distance. The package *rstatix* was used for pair-wise comparisons of the treatments for each ARG. This was done using Wilcoxon tests followed by the Benjamini-Hochberg test for false discovery rate ( $p \leq 0.05$ ).

## 2.6 Inoculation and analysis of *Trichoderma*

Inoculation of *Trichoderma* was performed two months prior to the experiment to allow enough time to stabilize in the system, by adding 12 g of spores to each bed (1000 g of spores /  $\text{m}^3$  of soil, i.e., the filtration layer). Inoculation was repeated three times with a 7-day interval in both June and July 2021. Throughout the stabilization period of the system and the sampling campaign, the population density of *Trichoderma* was assessed using the methodology outlined by López-López et al. (2016). This evaluation aimed to confirm its presence in the inoculated systems and its absence in the other beds. Namely, water samples from all systems were collected using sterile bottles and subjected to the dilution plating on semi-selective media for *Trichoderma* (Chung and Hoitink, 1990).

## 3. Results and discussion

### 3.1. Effect of bioaugmentation on conventional water quality parameters

CWs are a nature-based solution typically employed for secondary treatment. However, they can also be adopted for tertiary treatment to comply with more stringent water quality standards for discharge in the sensitive environment by providing an additional denitrification and carbon removal step. A reduction in dissolved organic carbon content in secondary treated wastewater effluents has

been shown to positively affect the biodegradation of CECs (Kahl et al., 2017). Thus, the tertiary treatment option was selected in this study. Table 1 shows average values of conventional water quality parameters in the influent and effluent of three CW lines: without plant, with plant, and with plant inoculated with *Trichoderma*.

Parameter	Influent	Effluent (control)	Effluent (plant)	Effluent (plant + T34)
NH <sub>4</sub> -N (mg/L)	2.12 ± 0.25	0.26 ± 0.04 (88%)	0.13 ± 0.03 (94%)	0.12 ± 0.03 (94%)
NO <sub>2</sub> -N (mg/L)	0.24 ± 0.05	0.12 ± 0.03 (50%)	0.04 ± 0.02 (83%)	0.04 ± 0.02 (83%)
NO <sub>3</sub> -N (mg/L)	12.38 ± 1.1	12.70 ± 1.1 (0%)	2.80 ± 0.5 (77%)	2.63 ± 0.5 (79%)
Total N (mg/L)	40.67 ± 8.5	30.67 ± 6.6 (25%)	23.67 ± 7.2 (42%)	20.06 ± 4.8 (51%)
PO <sub>4</sub> -P (mg/L)	0.90 ± 0.18	0.85 ± 0.22 (6%)	0.08 ± 0.1 (91%)	0.08 ± 0.1 (91%)
COD (mg/L)	51.25 ± 10.2	48.25 ± 7.8 (6%)	32.25 ± 6.4 (27%)	36.25 ± 9.2 (29%)
SO <sub>4</sub> <sup>2-</sup> (mg/L)	49.75 ± 8.9	51.25 ± 9.0	58.75 ± 10.2	58.50 ± 10.2
pH	7.9 ± 0.1	8.1 ± 0.1	7.8 ± 0.1	8.0 ± 0.1
EC (µS/cm)	1238 ± 271	1160 ± 248	1424 ± 128	1476 ± 142
DO (mg/L)	2.6 ± 0.8	1.2 ± 0.6	0.2 ± 0.2	0.2 ± 0.2

Table 1. Average concentrations (± SD) of conventional water quality parameters at the influent and effluent of partially saturated vertical subsurface constructed wetlands during September 2021 (n = 9) and removal efficiency (into brackets) without *Phragmites australis* (control), with *Phragmites australis* (plant) and with *Phragmites australis* inoculated with *Trichoderma* (plant + T34).

Nitrification was effective in all conditions, whereas denitrification occurred only in the presence of plants, likely due to the establishment of anoxic conditions, with an average DO value of 0.2 mg/L in effluents. This latter result accounted for the higher removal of TN in the presence of plants.

Additionally, nitrate ions can be assimilated in the roots and translocated to the aerial plant tissues for nitrogen incorporation into amino acids after consecutive reduction to nitrite and ammonium.

The presence of vegetation and microbial activity within CW units can contribute to both oxygen production and consumption. *Phragmites australis* is well known to transfer oxygen into the rhizosphere, however  $O_2$  distribution in the medium follows spatio-temporal oscillations that are directly linked with nutrient removal processes driven by root associated bacteria (Li et al., 2021). Differences of DO observed in unplanted units could thus be explained by changes in the diversity of microbial communities. Differences in total N removal observed between planted and unplanted units is related to particulate organic N microbial turnover which depends on the microbial community composition and diversity, known to increase with plant presence (Zhang et al., 2010). The effect of *Trichoderma* was clear (30.67 mg/L total N in control units vs 23.67 mg/L in planted units and only 20.06 mg/L in inoculated planted units). Whether *Trichoderma* contributed to this delta in N removal by direct assimilation or by modifying microbial community remains inconclusive and needs further studies. Similarly, complete removal of  $PO_4$ -P, occurring at low levels in the influent (< 1 mg/L), was only attainable in the presence of plants due to transformation by microbial communities associated with the plants and the plants' uptake of this nutrient. Previous studies have estimated that the uptake of phosphorus by plants in CW is minor (around 5%) and that the major removal comes from microbial removal and fixation in the soil filter (Stottmeister et al., 2003). The presence of plants also enhanced the removal of carbon due to the higher oxygen transfer capacity to the bottom layers of the CW through specialized tissue known as aerenchyma. Conductivity showed a slight increase in presence of plants due to a higher turnover of organic matter degradation by rhizospheric microbial communities and a concomitant increase of the availability of soluble macro and micronutrients. In particular, the increase of sulfate in planted wetlands may result from the plant-mediated oxygen transfer which increases the redox potential and inhibits the activity of sulfate-reducing bacteria. Seasonal impact on sulfur cycle has been observed in CWs, resulting in different  $SO_4^{2-}$  removal rates (Wu et al., 2020). All these results mostly agreed with previous studies involving similar CW systems (Avila et al., 2021a). However, this study found that redox conditions facilitated denitrification but not sulfate reduction, potentially limiting the diversity

of bacterial communities and the enrichment of sulfate-reducing bacteria. As a whole, inoculation with *Trichoderma asperellum* (strain T34) did not significantly improve the CW performances in terms of removal of nutrients and carbon.

### 3.2. Effect of bioaugmentation on CECs removal.

For the assessment of CECs treatment efficiency, a non-target LC-HRMS screening was implemented. Non-target screening generated reliable estimates of RE based on the peak area ratio in influent and effluent for a large number of contaminants covering the whole range of biodegradability and a wide range of polarity (Li et al., 2018). However, non-target screening did not preclude the use of indicator compounds that could be used as proxies for other chemicals of comparable behavior to assess the differences in matrix effects (i.e., influent vs effluent) in LC-HRMS analysis. This was done by collecting quantitative data with the use of deuterated internal standards. Effect-based methods have also been introduced in the evaluation of CW performances for CECs removal (Sosalla et al., 2020), providing a more comprehensive picture of the biologically active compounds contained in water samples than simple chemical analysis. However, this approach was discarded due to the forward-looking character of the work. Consequently, nine indicator compounds were selected, including benzotriazole, carbamazepine, clarithromycin, diclofenac, fluconazole, propranolol, sulfamethoxazole, tramadol, and venlafaxine (see Table 2) due to their consistent occurrence during the four weeks of study.

Compound	Frequency (%)	Influent concentration	RE (%) Plant	RE (%) plant + T34	RE (%) in studies with VF CW
Carbamazepine	100	234.9 ± 35.1	16.8 ± 54.5	15.4 ± 55.2	22 ± 30 <sup>a</sup> or 40 ± 20 <sup>b</sup>
Tramadol	100	248.5 ± 32.2	- 6.4 ± 32.4	- 7.3 ± 28.5	39 ± 43 <sup>a</sup> or 46 ± 42 <sup>b</sup>
Venlafaxine	100	209.4 ± 25.4	- 5.4 ± 14.0	-2.3 ± 16.7	36 ± 27 <sup>a</sup> or 40 ± 21 <sup>b</sup>
Fluconazole	100	95.6 ± 14.7	-23.1 ± 22.6	-12.9 ± 26.4	7 ± 29 <sup>a</sup>
Sulfamethoxazole	100	3888.3±1166.2	34.6 ± 18.2	39.7 ± 24.3	43 ± 42 <sup>a</sup> or 54 ± 29 <sup>b</sup>

Propranolol	100	47.6 ± 8.4	76.8 ± 36.2	75.9 ± 35.6	59 ± 31 <sup>a</sup>
Clarithromycin	100	80.4 ± 12.5	39.9 ± 20.2	49.8 ± 18.5	39 ± 33 <sup>a</sup> or 49 ± 57 <sup>b</sup>
Benzotriazole	100	531.6 ± 66.8	42.2 ± 10.2	56.5 ± 25.4	67 ± 24 <sup>c</sup>
Diclofenac	100	402.6 ± 48.8	77.7 ± 8.2	88.6 ± 8.8	38 ± 35 <sup>a</sup> or 50 ± 17 <sup>b</sup>

Table 2. Mean and standard deviation (%) of influent concentration (ng/L) and removal efficiency (RE in %) of indicator compounds in CW units without and with bioaugmentation with *Trichoderma* during four subsequent sampling weeks. a) values from Ilyas et al., 2020; b) values from Carvalho, 2021.

Their concentrations in influent ranged from a few tens of ng/L for the  $\beta$ -blocker propranolol to a few  $\mu\text{g/L}$  for the antibiotic sulfamethoxazole. High standard deviation values (from 8.8 to 55.2%) associated with RE were found. The high variability in RE is a common feature in CW studies (Ilyas et al., 2020; Carvalho, 2021) and remained the main constraint in treatment efficiency even though the fungus could grow under our operational conditions. The average number of colony forming units (CFU) of *Trichoderma* in effluent increased from  $167 \pm 22\%$  the first week of sampling to  $211 \pm 9\%$  and  $188 \pm 7\%$  the second and third week to reach  $808 \pm 14\%$  the fourth week, while no CFU was detected in CW units without inoculation. This result was an interesting finding because microbial strains with high pollutant degrading activity under controlled laboratory conditions frequently failed to exert their beneficial effects at the field-scale. This is possibly due to a lack of favourable habitat for their growth and the poor fitness of laboratory-grown inoculants to compete with indigenous microbial communities in polluted environments (Xiang et al., 2022). Added *Trichoderma* may promote plant performance by enhancing nutrient availability, and reducing plant stress response to contaminants, or act as biocontrol agents against native pathogens present in the growth substrate (Tondera et al., 2021). They may also promote plant contaminant uptake because they are endophytic fungi and may also promote CECs rhizodegradation through the external secretion of laccase and peroxidase enzymes (Manasfi et al., 2020). However, RE values of targeted indicator



compounds were mostly found lower than the ones reported in the literature. This held particularly true for tramadol and venlafaxine for which their RE values were found negative, while these latter exceed sometimes 50% in previous studies. For CWs in their first months of operation as it was the case in this study, the CECs biodegradation capacity might be underestimated, as the microbial communities develop this capacity over time in symbiosis with plant development (Wagner et al., 2023). However, the main objective was not to optimize one particular wetland treatment and to reach high RE values but to compare two operation modes (i.e., with and without bioaugmentation with *Trichoderma*). The use of secondary treated wastewater to feed the CW pilot might also account for these low RE values. In particular, negative RE by CWs can occur as a result of various processes such as variation in influent concentrations or deconjugation of TPs generated during the secondary treatment stage. In spite of this, after wetland treatment, only sulfamethoxazole concentration exceeded its PNEC value (0.6 µg/L) for freshwater as reported in the NORMAN ecotoxicology database, highlighting the need for stronger treatment processes for this specific compound. Biodegradation has been assumed to be the primary removal pathway in treatment wetlands (Avila et al., 2021a). Carbamazepine, venlafaxine and tramadol persisted in CW treatment. This is consistent with their known recalcitrance. Propranolol and clarithromycin were most likely removed by sorption processes due to their high log D values (3.5 and 3.2, respectively) while sulfamethoxazole is known to be biodegraded both under aerobic and anaerobic conditions (Wang et al., 2021). Inoculation with *Trichoderma* only had a clear beneficial effect on diclofenac and benzotriazole removal with an increase of over 10% in their mean RE values. Previous studies indicated low to moderate removal of diclofenac by most wetland treatment systems (38 to 50%, see Table 2). This is in contrast to the high RE of nearly 90% obtained in this study. However, the isolated *Trichoderma longibrachiatum* strain has also revealed a RE of 80% for diclofenac in liquid media in 1 d due to the production of ligninolytic enzymes resulting in hydroxylation reactions as the first step of the degradation pathway (Kasonga et al., 2021). One reason for the limited benefit of the action of external secreted enzymes (i.e., laccase and peroxidase) for the removal of other targeted organic

micropollutants might be ascribed to their quick flushing outside the treatment wetland, besides to their different affinities leading to competitive catalytic reactions. Increasing the hydraulic retention time (HRT) might allow slower transformation processes to occur to a larger extent. Benzotriazole has been found to be rapidly assimilated by the plants such as *Arabidopsis thaliana* followed and undergoes significant phytotransformation afterward. Active plant uptake has been hypothesized for benzotriazole since the uptake was greater than the passive rate of water uptake by evapotranspiration (Lefevre et al., 2015). Benzotriazole was also found to be eliminated through plant uptake and phytotransformation in bioretention cells (Gu et al., 2021). *Trichoderma spp.* are endophytic fungi that promote host plant growth and nutrient uptake, particularly under stress, by colonizing the root surface and by living as endophytes inside root tissues (Tsenget al., 2020). In these conditions, the higher plant growth rate could support the higher benzotriazole plant uptake. As expected, non-target LC-HRMS analysis of influent samples revealed that a very large number of chemicals were present in urban domestic wastewater when using positive mode of ionization. Chemicals were identified at the level of confidence 2 on the Schymanski scale by matching their spectrum with library spectrum data (i.e., mz cloud, Tadić et al., 2021). Most of them were pharmaceuticals and a large range of RE values were obtained among compounds (see Supplementary Excel file 2). Many of these contaminants are rarely reported and little is known regarding their transport and fate in biological treatment systems. Biodegradation together with sorption was found to be the main interlinked drivers for a successful RE. The interlinked impact of log Dow, as a good indicator of the hydrophobicity of both neutral and ionic organic compounds as well as the impact of compound biodegradability on RE is shown in Fig. 1.

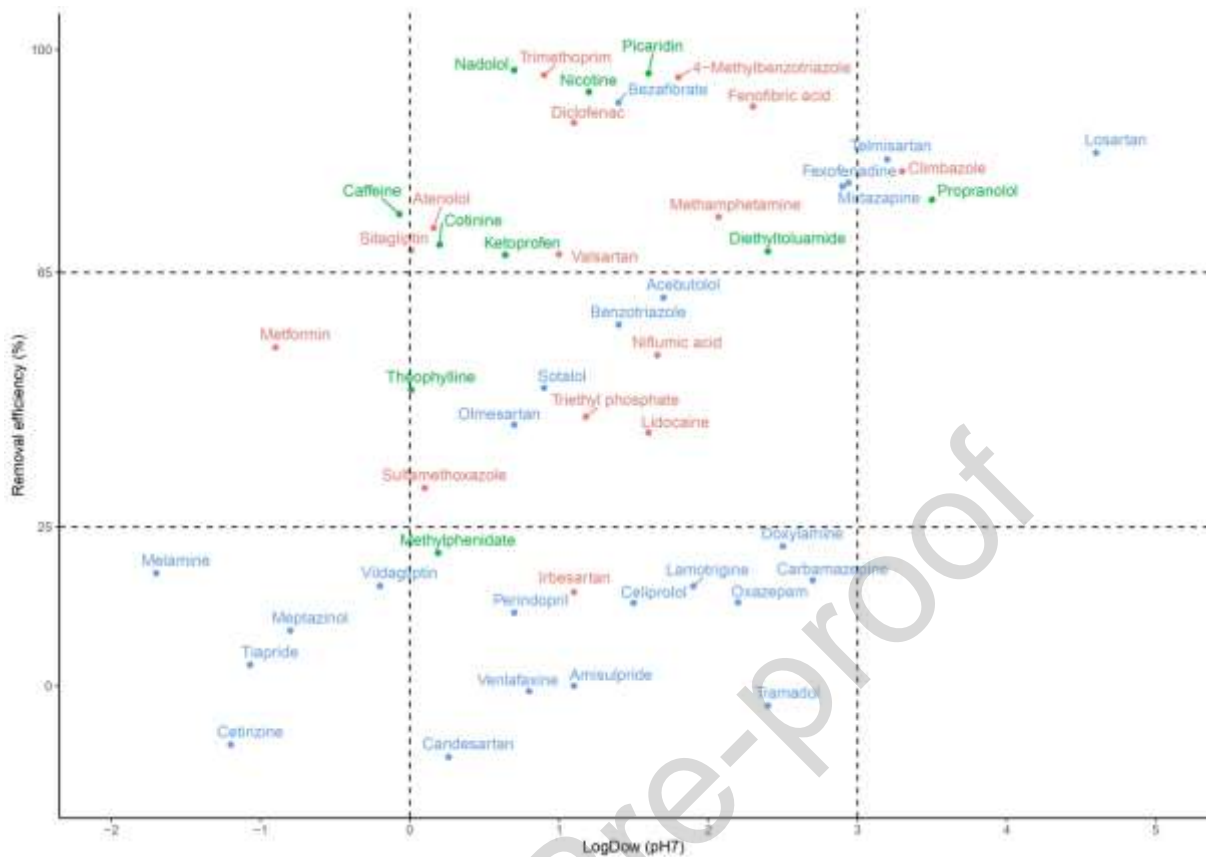


Fig. 1. Removal efficiency (in %) of contaminants of emerging concern as a function of their log Dow and their biodegradability in CW units with plant inoculated with *Trichoderma*. Different colors are used to classify the significance of each chemical biodegradability: Green color for quickly biodegradable compounds (dissipation time ( $DT_{50}$ ) < 2 d), blue color for moderately biodegradable compounds ( $2 < DT_{50} < 10$  d) and red color for slowly biodegradable compound ( $DT_{50} > 10$  d) following  $DT_{50}$  literature available data.

Due to a high range of variability in biodegradation rates found in the literature (mainly in activated sludge) and sometimes contradictions in findings, it was decided to sort the compounds biodegradability into three categories namely, quickly biodegradable compounds (dissipation time ( $DT_{50}$ ) < 2 d), moderately biodegradable compounds ( $2 < DT_{50} < 10$  d) and slowly biodegradable compound ( $DT_{50} > 10$  d). The three categories are depicted in three different colors in Fig. 1. In this way, low ( $RE < 25\%$ ), moderate ( $25 < RE < 65\%$ ) to high ( $RE > 65\%$ ) potential for successful treatment

of CECs were obtained. Firstly, quickly biodegradable compounds (green color) such as caffeine and nicotine were easily eliminated. More surprisingly, RE values higher than 65% were also observed for a few slowly biodegradable compounds (red color) such as 4-methylbenzotriazole, trimethoprim and diclofenac. Most of these compounds have a  $\log D_{ow}$  between 0 and 3, range that has been previously reported as optimal for xenobiotic plant uptake (Collins et al, 2011). Even though a scientific consensus has attributed to plant uptake a minor role in the RE of organic contaminants in large scale CW, some studies have shown a positive correlation between transpiration rate and RE (Carvalho, 2020). This was because, in small CW units, the evapotranspiration could represent a significant percentage in the water flow balance. In such conditions, the role of plant uptake in the removal of some compounds easily taken up could be significant. High evapotranspiration rates would also justify the observed negative removals of compounds neither easily taken up, nor biodegradable nor adsorbed. However, no data from plant tissues were generated in this study to confirm this hypothesis. Secondly, hydrophobic compounds ( $\log D_{ow} > 3$ ) were highly removed by sorption processes. For instance, as far as the sartan family is concerned, telmisartan and losartan were highly removed due to their  $\log D_{ow} > 3$  despite being slowly biodegradable compounds. In contrast, only a slight negative removal of candesartan and a positive removal of olmesartan were obtained. This aligns with their higher hydrophilicity, with  $\log D_{ow}$  values of 0.26 and 0.7, respectively. Finally, valsartan and irbesartan are classified as moderately biodegradable (blue color) and moderately hydrophilic ( $\log D_{ow} = 1.9$  and 1.1, respectively). Both could be biotransformed into valsartan acid through N-dealkylation oxidation followed by oxidative deamination (Posselt et al., 2020). However, valsartan was much more efficiently eliminated than irbesartan (RE = 68% vs RE = 13% as a mean value). This can be explained because the N-dealkylation reaction occurring in a heterocyclic ring (irbesartan) was more limited due to steric and electronic hindrances in comparison to the N-dealkylation of an aliphatic chain (valsartan). Thirdly, moderate hydrophobicity ( $0 < \log D_{ow} < 3$ ) and low molecular weight ( $MW < 500$ ) are often recognized as adequate properties for chemicals to move through cell membranes, facilitating plant uptake. However, this knowledge would deserve

to be somewhat qualified because contrasting results for RE among poorly biodegradable compounds were obtained. This is well illustrated with the family of  $\beta$ -blockers where one representative of this family had a RE > 65% (i.e., atenolol), three with RE between 25 and 65% (i.e., sotalol and acebutolol) and one with a RE < 25% (i.e., celiprolol). Nadolol and propranolol, two closely related chemical structures, are actually readily biodegradable compounds and easily eliminated, hence they are discarded from the discussion. The variation in biotransformation rates was most likely due to different functional groups in chemical structures. Atenolol is an amide-containing compound that undergoes fast hydrolysis. Sotalol and celiprolol are sulfonamide- and urea-containing compounds, respectively. Both functional groups undergo slower hydrolysis than the amide group accounting for slower biodegradation rates than atenolol. Acebutolol is also an amide-containing compound but with N substituted with an aromatic ring. The hydrolysis reaction was therefore hindered due to resonance (Rich et al., 2022). Finally, highly hydrophilic compounds ( $\log \text{Dow} < 0$ ) could be removed only if they are quickly biodegradable (e.g., caffeine ( $\log \text{Dow} = 0$ ) and cotinine ( $\log \text{Dow} = 0.2$ )). Otherwise, they exhibited very low removal rates (RE < 25%, e.g., melamine and candesartan). Surprisingly, metformin which is a very polar compound ( $\log \text{Dow} = -1.2$ ) and classified as a moderately biodegradable compound in activated sludge biodegradation test had a high RE of 52% (mean value). However, the biodegradation rate of metformin into guanyurea was probably underestimated in the activated sludge because fast biodegradation of metformin has also been observed in water/sediment system (Posselt et al., 2020).

As the influent was a secondary treated WWTP effluent, transformation products (TPs) were also investigated. A total of 22 TPs were identified by LC-HRMS in the influent following a workflow previously published by our group (see Supplementary Excel file 3 and Tadić et al., 2022). Tramadol N-oxide, valsartan acid, venlafaxine N-oxide, O-desmethylvenlafaxine N-oxide were confirmed with reference standards, whereas the others were confirmed by matching their spectrum with library spectrum data (i.e., mz cloud). A retrospective analysis of identified TPs was carried out in CW units effluent. For 16 TPs, mean values of the RE were positive (see Fig. 2a) mostly due to biodegradation,

whereas for 6 TPs, negative RE was estimated based on the peak area (see Fig. 2b) most likely due to their higher stability after formation during CW treatment.

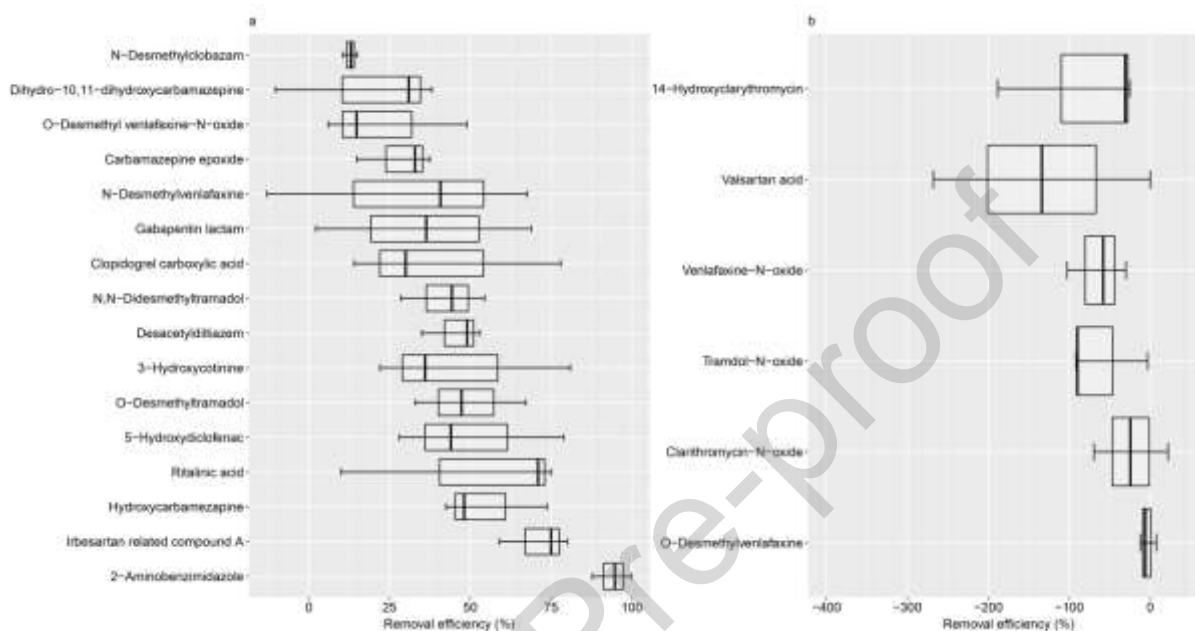


Fig. 2. a) Positive and b) negative mean values and standard deviations of removal efficiency (%) of selected transformation products of pharmaceuticals detected in influent during VSSF-CW treatment.

Identification of TPs helped in revealing the main transformation reactions involved in parent compounds removal. These transformation reactions comprised hydrolysis of esters (methylphenidate and clopidogrel) or acetylated compounds (diltiazem and carbendazim), N-demethylation (venlafaxine, tramadol, clobazam), hydroxylation (carbamazepine, diclofenac, clarithromycin, and cotinine) and N-oxidation (tramadol, venlafaxine, clarithromycin, and O-desmethylvenlafaxine). All these biotransformation pathways have already been found to occur in wastewater microbial communities (Rich et al., 2022). Most of the resulting TPs underwent further transformations/degradation even though with a high variability in intensity similarly to parent compounds. 2-aminobenzimidazole, the primary TP of the fungicide carbendazim, exhibited the most

straightforward removal in CW units, with a RE > 90%. Surprisingly, gabapentin lactam which is formed via intermolecular amidation reaction (lactamization) and is known to be a persistent TP as it can be detected in drinking water (Henning et al., 2018) could be further removed. Given amidation is a reverse reaction, gabapentin-lactam could revert to the parent compound during CW treatment, contributing to the extensive variability in gabapentin RE values. 5-Hydroxydiclofenac could be easily further oxidized into the toxic benzoquinone-imine derivative. Transformation of O-desmethyltramadol probably involved N-dealkylation to give N-,O-didesmethyltramadol. As expected, more recalcitrant TPs were 10, 11-dihydroxycarbamazepine, O-desmethylvenlafaxine-N-oxide and, N-desmethylclobazan. This latter TP has a chemical structure closely resembling oxazepam, known for its high resistance to biodegradation. Among TPs that were able to be formed in CW are the N-oxidized TPs of tertiary amines including venlafaxine N-oxide, tramadol-N-oxide and clarithromycin N-oxide. It is known that N-oxidation reactions constitute a major microbial biotransformation pathway for alkyl tertiary amines. The stability of N-oxides TPs in CW is consistent with our previous findings on the stability on N-oxides in surface water (Manasfi et al., 2022). Moreover, observed transformation reactions do not necessarily deactivate the molecule; instead, they render it suitable for biological activity. For instance, 14-hydroxycarithromycin, which was also formed during treatment is still pharmacologically active, hence might contribute to the selective pressure on bacteria. Similarly, carbamazepine-epoxide, which was only partially removed in CW, was proven to be more toxic than carbamazepine. While the importance of the O-desmethylvenlafaxine occurrence has been already recognized, hence this TP was added to the 3<sup>rd</sup> EU Watch List due to its persistence and toxicity (Maculewicz et al., 2022). Finally, the common transformation pathway of several sartans into valsartan acid including irbesartan and valsartan accounted for the higher concentration of valsartan acid in the effluent than in the influent (Ladhari et al., 2021).

### 3.3. Effect of bioaugmentation on ARGs removal.

The 16S rRNA gene copy numbers, used here as a proxy for bacterial abundance (Table S2), ranged from  $3.29 \times 10^{12}$  to  $4.21 \times 10^{13}$  16S rRNA copies/mL. The values between groups were not significantly different (Table S3), showing that there was not a reduction of bacterial abundance from the influent to the effluent samples. Ávila et al.(2021b) observed similar results regarding the 16S rRNA gene copy numbers, using CW operating with different configurations.

Out of the 72 genes targeted in our qPCR analysis, only 56 genes were identified in both influent and effluent samples, including *int11*, *sul1*, *sul2*, *ermB*, *blaTEM*, and *qnrS* (see Supplementary Excel file 1).

The relationship between ARGs and sample types was analyzed by PCA. The first PCA component, PC1, explaining 54.7 % of the total variance, differentiated the inlet samples from the plant and plant + T34 samples (see Fig. 3). Meanwhile, the second component, PC2, accounting for 20.2 % of the total variance, broadly distinguished samples from plant and S-plant + T34 experiments, represented by red and grey circles.



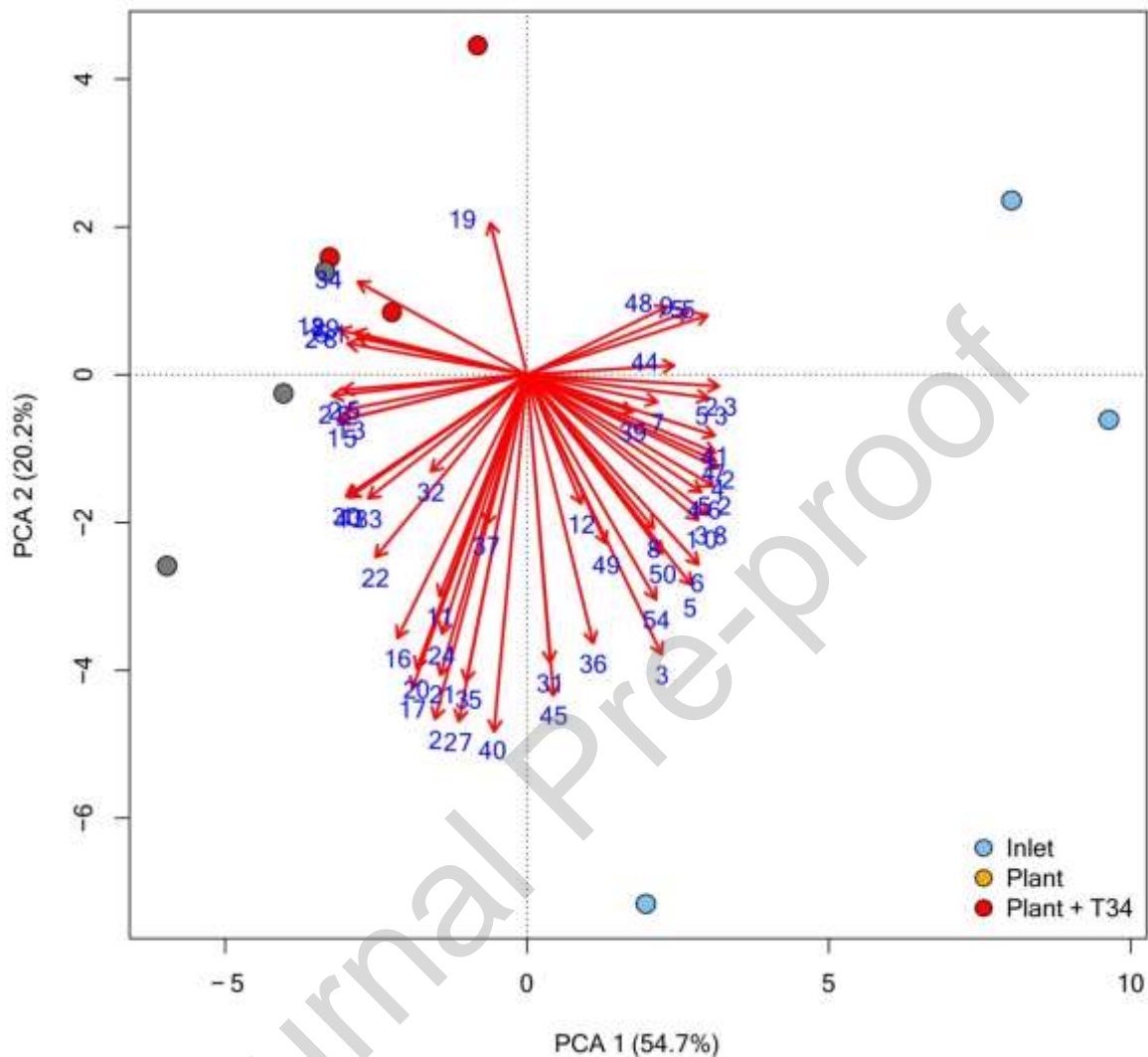


Fig. 3. PCA plot for the analysis of the distribution of ARGs among sample types. Sample types are represented by different colors. Blue, red, and grey circles correspond to inlet, CW with plant, and CW with plant + *Trichoderma* samples, respectively. ARGs loadings are represented by the in blue with a red arrow. The plot corresponds to the two first components, the percentage of variance with the total variance of the two components presented in the plot. Each number corresponds to one ARG as explained in Supplementary Material.

The loading plots indicated that ARGs were more associated with inlet and plant + T34 samples. A total of 55 ARGs were detected in the inlet, whereas 47 were found in both CW with plants and those

inoculated with *Trichoderma*. These results are in concordance with Liu et al. (2020). In their study, the authors showed there was a higher number of ARGs in the inlet than in the outlet, indicating a higher diversity. The variation in oxygen levels, as shown in Table 1 (ranging from an average dissolved oxygen value of 2.6 to 0.2 mg/L in effluents), might contribute to the variability in bacterial diversity (Aldunate et al., 2018) and consequently the ARGs diversity (Yu et al., 2020, Quintela-Baluja et al., 2019). The diversity of ARGs was analyzed, either intra-sample ( $\alpha$ -diversity) or between-sample diversity ( $\beta$ -diversity).  $\alpha$ -diversity indexes did not indicate any significant differences between the sample types (Table S4). Though the variation between samples visualized by a NMDS (Fig. S1) revealed distinct groups of samples using the Jaccard distance, consistent with the PCA analysis. There was a much higher dissimilarity for ARGs between inlet samples and the plant and plant + T34 experiments, than between the latter two. The contribution of the defined groups *a priori* on the ARGs composition was tested by the *adonis* PERMANOVA (Supplementary Table S5), confirming significant differences in ARGs composition between sample types, either considering variations in abundance (Bray-Curtis,  $p=0.04$ ) or presence/absence (Jaccard,  $p=0.01$ ) of ARGs. Both cases explained approximately 60% of the ARGs variance, either in ARGs composition changes with Bray-Curtis (0.603) and ARGs turnover with Jaccard (0.596). Following  $\beta$ -diversity, we focused on finding ARGs that could explain the difference in the composition shift and turnover among the different sample types. However, no significant differences were found in the abundance of individual ARGs between the sample types (Supplementary Table S7). We interpreted these results as a demonstration that the sample types (different treatments), may have caused a shift in the ARGs composition and turnover. However, that effect is limited as there were no significant differences in the ARGs abundance between groups. Avila et al. (2021b) and Sabri et al. (2021) showed similar results that the removal efficiency of ARGs from CW was not significant or low.

## Conclusions

This study provided additional data on the capacity of bioaugmentation of VSSF- CW units with *Trichoderma* to eliminate a large array of CECs. Overall, the RE values were suboptimal because the investigated CW units were in their initial months of operation, potentially leading to an underestimation of the CECs biodegradation capacity. The most easily eliminated compounds were those which are known to be readily biodegradable (e.g., nicotine). Notably, out of 22 compounds with RE > 65%, 17 had a log Dow between 0 and 3, indicating optimal plant uptake. The most difficult compounds to be eliminated were the most polar ones (log Dow < 0) such as melamine. Our treatment wetland also generated TPs. Interestingly, N-oxide TPs of tertiary amines and 14-hydroxyclearithromycin were frequently detected in effluent. The attempts at bioaugmentation with *Trichoderma* were successful, as the fungus could thrive in competitive conditions. However, the high range of standard deviation values associated with RE values resulted to be the main issue for a proper assessment of treatment efficiency. This calls for more fundamental research into the mechanisms of compound attenuation to find strategies to limit fluctuating RE. Finally, neither of the wetland treatments (with or without bioaugmentation) significantly reduced the overall bacterial and ARGs loads. Hybrid treatment systems combining CW and advanced oxidation processes are currently implemented in our research group to avoid antibiotic resistance spreading.

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#### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Highlights

► Bioaugmented wetland with *Trichoderma* was evaluated by non-target LC-HRMS screening  
► *Trichoderma* was able to grow in real competitive conditions ► Removal efficiency higher than 65% was observed for slow biodegradable contaminants ► N-oxide TPs and 14-hydroxycarithromycin were formed in treatment wetland ► Treatment wetland failed to reduce the overall bacterial and ARGs loads