Towards Sustainable Wastewater Treatment: Bioindication as a Technique for Supporting Treatment Efficiency Assessment

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Abstract: Constructed wetlands (CWs) are a promising alternative for conventional methods of wastewater treatment. However, the biggest challenge in wastewater treatment is the improvement of the technology used so that it is possible to remove micropollutants without additional costs. The impact of wastewater treatment in CWs on toxicity towards *Alivibrio fischeri*, *Daphnia magna* and *Lemna minor* was investigated. The effects of feeding regime (wastewater fed in five batches per week at a batch volume of 1 L, or twice per week at a batch volume of 2.5 L) and the presence of pharmaceuticals (diclofenac and sulfamethoxazole), as well as the presence of *Miscantus giganteus* plants in CW columns (twelve of the 24 columns that were planted) were analyzed. A reduction in toxicity was observed in all experimental setups. The effluents from constructed wetlands were classified as moderately toxic (average TU for *A. fischeri*, *D. magna* and *L. minor* was 0.9, 2.5 and 5.5, respectively). The feeding regime of 5 days of feeding/2 days of resting resulted in a positive impact on the ecotoxicological and chemical parameters of wastewater (removal of TOC, N-NH4 and pharmaceuticals). Extended exposure of *Miscantus giganteus* to the wastewater containing pharmaceuticals resulted in elevated activity of antioxidant enzymes (catalase and superoxide dismutase) in leaf material.

Keywords: environmental depollution; removal of emerging contaminants; bioindication; constructed wetlands; ecotoxicity; micropollutants; pharmaceutical pollution

1. Introduction

Wastewater treatment plants based on conventional activated sludge technology or sequencing batch reactors and membrane bioreactors are not cost-efficient, energy balanced or sufficiently effective enterprises to remove micropollutants [1,2]. Constructed wetlands (CWs) are a promising alternative for conventional methods of wastewater treatment. CWs are artificial habitats made up of highly heterogeneous microbial communities and plants [3,4]. They provide significant water quality benefits and have a positive impact on wildlife habitat. CWs preserve and boost local biodiversity and can drive sustainable development [5–7]. The number of hydrophyte treatment plants increased significantly in the 1990s, and their use expanded to include the treatment of various types of wastewater (e.g., industrial). CWs have been used to restore habitat for native and migratory wildlife species, dispose of anthropogenic wastewater, control stormwater runoff, remediate brownfields and mitigate ecological disturbances resulting from the loss of naturally occurring wetlands. In addition, artificial wetlands have gained attention because of their economic and ecological benefits. Compared to conventional treatment technologies, CWs have proven to be an attractive and sustainable alternative due to their low costs and energy savings. Additionally, they provide natural habitats in urban and suburban areas, enhance aesthetic value in the local natural environment and are a favorable solution for small and...
medium-sized cities due to their easy operation and maintenance. Artificial wetlands are now widely used in many European countries [8,9].

Constructed wetland is a low-cost treatment technology; the operational and maintenance cost in constructed wetland is 1–2% of capital cost as compared to other technologies. Table 1 presents selected operational and maintenance costs attributed to CWs in comparison to SBR technology.

| Type of Costs            | Unit                  | CWs       | SBR       |
|-------------------------|-----------------------|-----------|-----------|
| Electricity             | KW year\(^{-1}\)      | 260,000   | 7,500,000 |
| Labor cost              | Full-time equivalent year\(^{-1}\) | 0.75      | 12        |
| Maintenance cost        | DFC year\(^{-1}\)     | 0.9       | 1.9       |
| Miscellaneous cost      | USD year\(^{-1}\)     | 26,000    | 245,000   |
| Impact on ozone depletion | kg CPC               | 6.6 × 10\(^{-9}\) | 6.6 × 10\(^{-7}\) |
| Land requirement        | m\(^2\) person\(^{-1}\) | 1.5–2.5   | 0.05–0.01 |

In general, constructed wetlands can be used to treat various types of wastewater with slightly lower removal efficiency and higher space requirements compared to other wastewater treatment technologies. Considering the low environmental impact of this type of facility and the elimination of the use of chemicals in the treatment process, as well as the lack of continuous supervision by the operator, this technology is definitely considered sustainable. However, the biggest challenge in wastewater treatment is the improvement of the technology used so that it is possible to remove micropollutants without incurring high additional costs. Micropollutants, such as pharmaceuticals, in household wastewater treatment are very difficult to remove and may exhibit a negative environmental impact [11]. The presence of micropollutants in the natural environment is largely due to aquatic reservoirs receiving treated wastewater [12,13]. Two of the most frequently detected pharmaceutical compounds in wastewater, surface water and groundwater are diclofenac (DCF) and sulfamethoxazole (SMX) [14]. DCF is a commonly used as a nonsteroidal anti-inflammatory drug (NSAID). Painkillers and anti-inflammatory drugs are the most commonly detected pharmaceuticals and personal care products (PPCPs) in wastewater due to their high consumption and over-the-counter availability [15]. In the European Union, DCF was included in the first watch list (WL) under the Water Framework Directive (WFD) [16,17]. The goal of the WL is to collect high-quality Union-wide monitoring data on potential water pollutants for the purpose of determining the risk they pose, and thus whether environmental quality standards (EQS) should be set for them at the EU level [18]. SMX is an antimicrobial sulfonamide used to suppress a wide spectrum of bacterial infections in human and veterinary medical practice [19]. The presence of SMX in the natural environment may be connected with bacterial resistance to antibiotics [20,21]. Moreover, SMX is one of the most often-detected xenobiotics in European wastewater treatment plants’ (WWTPs) effluents. According to the Felis et al. [20] the detection frequency for SMX in the effluents of 90 European WWTPs was 83%. The environmental risks resulting from the presence of pharmaceutical substances in wastewater emphasize a need for stringent monitoring of treated wastewater quality. However, current analytical procedures are complex, multistage and require sensitive measuring equipment. In practice, it means that the detection and determination of selected impurities, and in particular their transformation products (which may have higher toxicity than the parent compound), is not always possible. A more accessible and sensitive approach is needed for the monitoring of wastewater released to surface waters, and one that can monitor more than the standard physicochemical parameters.

Various strategies are used to regulate pharmaceutical residues in the environment [22]. The main technique is to monitor the concentrations of micropollutants in surface water, groundwater or treated sewage; however, as it was said before, these methods are often unable to detect all of the degradation products and are excessively expensive [23]. Another
promising strategy might be bioindication—the use of aquatic organisms to monitor the safety level of sewage [24]. It seems reasonable to assume that wastewater, which does not pose a risk to natural organisms, can be safely discharged into the environment. Therefore, it is worth focusing not only on the detection of micropollutants in the environment but also on the assessment of the environmental risk caused by these substances.

Bioindication can be used to assess the ecotoxicological properties of wastewater and the effectiveness of the treatment process. The recommended procedure for the evaluation of environmental samples is to perform ecotoxicity tests with at least three bioindicators. Several studies have described the use of three model species representing different classes of bioindicator organisms: *Lemna minor*, representing “producers”; *Daphnia magna*, representing “consumers”; and *Aliivibrio fischeri*, representing “decomposers”. The *L. minor* plant is a macrophyte that is considered a useful bioindicator for ecotoxicological studies due to its small size, fast growth rate and sensitivity to numerous contaminants. The most favorable habitat for *L. minor* is water rich with decomposing organic matter, ensuring a constant supply of nutrients and trace elements [25,26]. The freshwater crustacean *D. magna* inhabits a variety of aquatic environments globally [27] and is widely used to monitor environmental pollution. Crustaceans are very important organisms from an ecological perspective due to their propensity for cleaning water reservoirs from algae, bacteria and protozoa. The luminescent, Gram-negative marine bacterium *A. fischeri* is commonly used in ecotoxicological studies [28]. The amount of light produced by *A. fischeri* is proportional to its metabolic activity, and any decrease in enzymatic activity results in inhibition of bioluminescence [29].

The aim of this investigation was to verify if the selected bioindication methods would be sustainable to determine the environmental risk caused by the presence of pharmaceuticals (DCF and SMX) in wastewater. For this purpose, the effect of raw and treated wastewater on three aquatic model organisms: *L. minor*, *D. magna* and *A. fischeri* were evaluated.

2. Materials and Methods

2.1. Tested Pharmaceuticals: Diclofenac and Sulfamethoxazole

DCF and SMX were purchased from Sigma-Aldrich (St. Louis, MA, USA) (purity > 99%). The properties of the tested pharmaceuticals as well as their environmental concentrations are listed in Appendix A—Table A1.

2.2. Experimental CW System

The laboratory system of vertical flow CWs consisted of 24 columns (each with a diameter of 0.2 m and height of 0.8 m, Figures 1 and 2). In order to verify the effect of plants on the efficiency of wastewater treatment, including the removal of pharmaceuticals, half of the CW columns were planted with *Miscantus giganteus* marsh plants. The effect of the wastewater feeding regime, the presence of pharmaceuticals DCF and SMX (in concentrations 2 mg L$^{-1}$ each) and the presence of *M. giganteus* on wastewater treatment processes, as well as the impact of these factors on ecotoxicity parameters, were investigated. The CW columns were set up in a heated room where the temperature was kept constant between 22–25 °C. The temperature was not adjusted during the study.
The studies presented in this work are a continuation of the preliminary research published by Sochacki et al. [30]. The results obtained in the preliminary studies were promising; therefore, it was decided to increase the concentration of pharmaceutical substances (from 0.5 to 2 mg L$^{-1}$). Higher concentrations of the analyzed pharmaceuticals may occur in point sources of contamination, which undoubtedly are wastewater from single houses or from hospitals [15,20]. Since environmental micropollutants are usually detected in combination, a binary mixture (MIX) of DCF and SMX was prepared. The synthetic domestic wastewater was prepared in tap water according to a modified protocol previously described by Nopens et al. [31] (Appendix A—Table A2). Wastewater samples were collected within two months after obtaining the stable operation of the systems. Each
sample was analyzed separately in three technical replicates. The types of columns used in this experimental system, with assigned symbols, are listed in Table 2.

Table 2. Types of columns used in the experiment.

| Types of Columns | Feeding regime (R) | R1 | R2 |
|------------------|-------------------|----|----|
|                  | R1-CTRL-Planted   | R1 | R2 |
|                  | R1-PhC-Planted    | 2 days of feeding/5 days of resting | 5 days of feeding/2 days of resting |
|                  | R1-CTRL-Unplanted | 2.5 L/d | 1 L/d |
|                  | R1-PhC-Unplanted  | 5 L/week | 5 L/week |
|                  | R2-CTRL-Planted   | 0.08 m3 m−2 d−1 | 0.032 m3 m−2 d−1 |
|                  | R2-PhC-Planted    | yes | yes |
|                  | R2-CTRL-Unplanted | no | no |
|                  | R2-PhC-Unplanted  | no | yes |

1 Hydraulic loading rate; 2 pharmaceuticals (DCF and SMX); CTRL columns—columns fed with synthetic wastewater; PhC columns—columns fed with synthetic wastewater containing DCF and SMX.

2.3. Chemical Analysis of Wastewater

The measured chemical parameters included DCF and SMX concentration, ammonium nitrogen (N-NH₄, mg L⁻¹) and total organic carbon (TOC, mg L⁻¹) concentration. Previous study had indicated that these parameters had the highest impact on the wastewater treatment process [32]. The procedure used for analysis is described in Appendix A, Appendix A.1.3.

2.4. Activity of Antioxidant Enzymes in M. giganteus

For plant material enzymatic assays, a Pro200 homogenizer (Pro Scientific Inc., Oxford, CT, USA) was used to prepare leaf homogenates. Three similar-sized leaves of *M. giganteus* were taken from each test column after 241–290 days of experiment. For determination of catalase (CAT) activity, 0.06 M sodium phosphate buffer (pH 7.4) was added to homogenates, while 0.05 M carbonate buffer (pH 10.2) was added to determine superoxide dismutase (SOD) activity. CAT activity was determined using a static method described by Göth [33], while SOD activity was measured using method described by Misra and Fridovich [34]. The homogenates were centrifuged (20 min, 4000 rpm, 4 °C) and then stored at −45 °C until the antioxidant enzyme activity assays were performed.

Measurement of protein quantity and enzymatic activity in the samples was performed using an Evolution 220 spectrophotometer (Thermo Fisher Scientific, Waltham MA, USA) and Insight 2 software (2014) (Thermo Fisher Scientific, Waltham, MA, USA). Before measuring CAT and SOD enzymatic activity, the protein content in the samples was determined using the Bradford method [35]. The protein concentration of each prepared extract was determined with reference to a standard curve, using bovine albumin as the standard protein. The detail procedure used for enzymatic analysis is described in Appendix A, Appendix A.1.4.

2.5. Toxicity Tests towards Aquatic Organisms

Toxicity of samples towards *A. fischeri* (Modern Water, York, UK) was determined according to ISO 11348:3:2007 [36] using synthetic sea water, prepared in accordance with ISO 10253:2016 standards, as a control solution [37]. Microelements present in municipal wastewater (MgCl₂, Na₂SO₄, CaCl₂ or KCl) intensify the activity of microorganisms; therefore, a stimulatory response may be observed. The use of synthetic seawater eliminated the hormetic effect and increased bacterial sensitivity to the tested substances [38]. The composition of synthetic sea water is presented in Appendix A—Table A3. Inhibition of *A. fischeri* luminescence was determined (Microtox M500, Modern Water) after 15 min incubation and each assay was performed in 3 replicates.
Toxicity of samples toward *D. magna* was determined using the Daptoxkit F Magna kit (Microbiotest Inc., Gent, Belgium). The toxicity tests were performed in accordance with OECD 202 [39]. The tests were performed in triplicate and the effect concentrations (EC$_{50}$) were determined using logarithmic-probit and regression analysis methods.

The *L. minor* toxicity test was performed in accordance with OECD 221 [40] procedure in 3 replicates. After 7 days of incubation, the number of fronds was counted, and based on these data, the average growth rate ($\mu_{i-j}$) and the percentage inhibition of average growth rate ($\% I_r$) for each solution were calculated based on Equations (1) and (2), respectively:

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

(1)

$$\% I_r = \frac{\mu_C - \mu_T}{\mu_C} \cdot 100$$

(2)

where $N_{i,j}$ is the number of fronds in time $i$ ($j$); $t$ is the time between $i$ and $j$; $\mu_C$ is the mean $\mu$ value for the control solution; and $\mu_T$ is the mean $\mu$ value for the tested concentration.

### 2.6. Toxicity Classification of Wastewater

The toxic unit (TU) value classification [41] was used to determine the toxicity of wastewater. The TU values for each test organism were calculated as per Equation (3):

$$TU = \frac{1}{EC_{50}(IC_{50})} \cdot 100$$

(3)

The classification of wastewater according to the TU value is presented in Appendix A—Table A4. When the toxic effect of the undiluted sample of wastewater was in the range of 10–49%, the TU value was calculated based on Equation (4) [42]:

$$TU = 0.02 \cdot E$$

(4)

where $E$ is the toxic effect of the undiluted sample of wastewater on the test organism.

If the toxic effect of the undiluted sample of wastewater is lower than 10%, the TU takes the value 0 [41].

### 2.7. Statistical Analysis

Statistical tests were performed using STATISTICA 13 software (StatSoft Poland, Kraków, Poland). Statistical tests in environmental studies are an indispensable part of the analysis of results, since we usually have to deal with natural variability in results related to, for example, genetic variation or physiological fluctuations. The first step in the statistical analysis was to verify the normal distribution of the results obtained. This is an important step, as it allows for the selection of an appropriate statistical test for assessing the significance of differences between variants of the experiment. In the case of our study, we demonstrated the significance of differences between the effects induced by control and pharmaceutical-containing wastewater, as well as verified whether differences in the way that R1 and R2 columns are fed have a significant effect on the effect of pollutant removal, toxicity of treated wastewater and enzyme activity in wetland plants (*Miscanthus giganteus*). Normality of the data was tested using the Shapiro–Wilk test. For data sets with normal distribution, Student’s $t$-tests were used ($\alpha = 0.05$). Otherwise, the data were analyzed using the Mann–Whitney U test. The differences were considered statistically significant if $p < 0.05$. The Kolmogorov–Smirnov (K-S) test ($\alpha = 0.025$) was used to analyze the calculated HC$_5$ values.
3. Results
3.1. Chemical Analysis of Wastewater

Wastewater treatment was assessed based on standard chemical parameters such as TOC (Appendix A—Figure A1) and N-NH\textsubscript{4} concentration (Appendix A—Figure A2), as well as on efficiency of removal of pharmaceutical water contaminants (Appendix A—Figure A3). These parameters were determined both in the inflow and outflow from the CWs. The achieved removal efficiency of chemical parameters is presented in Table 3.

| Types of Columns | Removal Efficiency, % * |
|------------------|-------------------------|
|                  | TOC                     | N-NH\textsubscript{4} | DCF            | SMX            |
| R1-CTRL-Planted  | 87.8 ± 3.93             | 19.9 ± 7.3             | –              | –              |
| R1-PhC-Planted   | 87.3 ± 1.94             | 18.0 ± 11.4            | 68.8 ± 8.2     | 79.1 ± 4.3     |
| R1-CTRL-Unplanted| 90.8 ± 1.08             | 39.3 ± 10.3            | –              | –              |
| R1-PhC-Unplanted | 90.0 ± 0.97             | 22.9 ± 12.6            | 13.7 ± 16.3    | 78.9 ± 22.5    |
| R2-CTRL-Planted  | 93.3 ± 1.43             | 45.8 ± 11.9            | –              | –              |
| R2-PhC-Planted   | 92.3 ± 1.12             | 58.9 ± 10.0            | 86.8 ± 9.7     | 98.0 ± 0.8     |
| R2-CTRL-Unplanted| 93.7 ± 1.07             | 29.1 ± 8.3             | –              | –              |
| R2-PhC-Unplanted | 94.1 ± 0.59             | 58.3 ± 4.6             | 76.6 ± 9.4     | 97.4 ± 0.7     |

* The removal efficiency (R) was calculated based on the influent and effluent concentrations according to 
  \[ R(\%) = \frac{C_{\text{influent}} - C_{\text{effluent}}}{C_{\text{influent}}} \times 100. \]

More than 87% of TOC was removed in all test columns (Table 3). However, the feeding frequency had an impact on the rate of TOC removal by the CWs. Higher removal rates were observed for R2 columns (feeding regime of 5 times a week in a volume of 1 L) with lower HLR. The presence of \textit{M. giganteus} in the test columns did not have a significant effect on TOC removal. \textit{M. giganteus} is a plant used as an energy crop and is characterized by rapid growth, even in poor soils. It can also be used in the CW wastewater treatment process [43]. In the case of N-NH\textsubscript{4}, the removal rate ranged from 18.0 to 58.9% (Appendix A—Figure A2; Table 3), and the presence of plants also did not have a statistically significant impact on the removal of this parameter. However, the removal of N-NH\textsubscript{4} was influenced by the frequency of wastewater dosing and the presence of pharmaceuticals. More efficient removal of TOC and N-NH\textsubscript{4} was observed from wastewater applied to R2 columns (feeding frequency of 5 times a week in a volume of 1 L) (average removal for TOC = 93.4% and for N-NH\textsubscript{4} = 48.0%) compared with their removal from wastewater dispensed to R1 columns (feeding frequency of twice per week at a volume of 2.5 L) (89.0% and 25.0%, respectively). Thus, it could be seen that the frequency of wastewater dosing had the greatest impact on the removal of both organic and nitrogen compounds.

DCF removal efficiency in all analyzed samples was lower than SMX removal (Appendix A—Figure A3; Table 3). The frequency of wastewater dosing was found to affect the removal efficiency of DCF and SMX. Better removal of both pharmaceuticals was observed in the effluent from R2 columns compared to the effluent from R1 columns, which was confirmed by statistical analyses (Appendix A—Figure A3). It was observed that the presence of \textit{M. giganteus} in CW influenced the removal of DCF in all columns tested, while in the case of SMX, the presence of plants influenced its removal only in R2 columns.

3.2. Antioxidant Enzymes in \textit{M. giganteus}

The activity of CAT and SOD in \textit{M. giganteus} are presented in Figure 3. The effect of the presence of pharmaceuticals and the frequency of wastewater dosing on the antioxidant enzymes’ activity in \textit{M. giganteus} plants was observed (Figure 3, Appendix A—Table A5). Higher activity of these enzymes was noted in plants from R2 columns.
(feeding frequency of 5 times a week in a volume of 1 L). CAT activity in plants from R1 columns fed with control wastewater averaged 136.7 μmolH2O2 min−1 mgprotein−1, while that in plants from R2 columns fed with the same type of wastewater averaged 169.7 μmolH2O2 min−1 mgprotein−1. A similar phenomenon was observed for columns fed with wastewater containing pharmaceuticals. In plants from R1 columns, the average CAT activity was 165.3 μmolH2O2 min−1 mgprotein−1, while an increase in CAT activity was also observed in plants from R2 columns, with an average of 205.9 μmolH2O2 min−1 mgprotein−1.

As the statistical analyses showed, the differences between CAT activity in plants from R1 and R2 columns were statistically significant with α = 0.05.

![Graph A](image)

![Graph B](image)

**Figure 3.** Activity of CAT (A) and SOD (B) in *M. giganteus*. (a) Statistically significant differences between counterpart types of columns (CTRL or PhC) with different frequency of wastewater dosing (Student’s t-test, α = 0.05); (b) statistically significant differences between control wastewater and wastewater containing pharmaceuticals (Student’s t-test, α = 0.05).

In the case of SOD, an increase in the activity of this enzyme was observed in leaf samples taken from R2 columns compared to leaf samples taken from R1 columns. For samples taken from columns fed with control wastewater, the average activity of SOD was 37.2 U min−1 mgprotein−1 for columns from the R1 system and 46.1 U min−1 mgprotein−1 for columns from the R2 system. Statistical analysis showed that for the control wastewater, the difference in SOD activity between R1 and R2 was statistically significant, with α = 0.05.

For leaf samples taken from columns fed with pharmaceutical-containing wastewater, SOD activity was higher than for samples taken from columns fed with control wastewater (R1: average SOD activity 52.4 U min−1 mgprotein−1, R2: average SOD activity 59.6 U min−1 mgprotein−1). The observed differences were statistically significant only in comparison to the control wastewater.

Differences in SOD activity in leaf samples taken from R1 and R2 columns fed with pharmaceutical-containing wastewater were statistically insignificant. We believe that the method of feeding influences SOD activity (which was proven in the case of control wastewater), but the introduction of an additional stress factor, such as the presence of pharmaceuticals, masked this effect. The likely reason for the lack of statistically significant differences in the case of SOD is the masking of the toxic effect by a combination of...
stressors. It is also worth noting that the presence of CAT will be a more sensitive indicator of oxidative stress in *M. giganteus*. The main reason for the observed dissimilarity between the enzymes activity under stress conditions is the different mechanism of action of CAT and SOD. This was the first observation of this kind of relationship between operating parameters of CWs and activity of antioxidant enzymes in *M. giganteus*.

### 3.3. Toxicity of Wastewater

In Figure 4, the TU values, indicating toxicity of the wastewater toward *A. fischeri*, *D. magna* and *L. minor*, are shown. A decrease was observed in wastewater toxicity after treatment toward all organisms except *A. fischeri*.

![Box plots showing toxicity values](image)

**Figure 4. Cont.**
Figure 4. Toxicity of wastewater to aquatic organisms: (A) A. fischeri; (B) D. magna, 24 h; (C) D. magna, 48 h; (D) L. minor. (a) Statistically significant differences between the wastewater influent and effluent (Student’s t-test, α = 0.05); (b) statistically significant differences between counterpart types of columns (CTRL or PhC) with different frequency of wastewater dosing (Student’s t-test, α = 0.05); (c) statistically significant differences between control wastewater and wastewater containing pharmaceuticals (Student’s t-test, α = 0.05); (d) statistically significant differences between wastewater from planted columns and from columns without plants (Student’s t-test, α = 0.05).

Based on these data, the average wastewater toxicity reduction (WTR, %) was calculated according to Equation (5) and is presented in Appendix A—Table A6.

\[
WTR = \frac{(TU_{IN} - TU_{EF}) \cdot 100}{TU_{IN}}
\] (5)
where $TU_{IN}$ is the average toxic unit calculated for influent wastewater and $TU_{EF}$ is the average toxic unit calculated for effluent wastewater.

Wastewater toxicity reduction (Appendix A—Table A6) was higher for R2 columns (average WTRs toward $A. fischeri$ and $D. magna$ after 48 hrs and $L. minor$ were 43.8, 52.0 and 73.9%, respectively) in comparison to R1 columns (average WTRs toward test organisms were 22.3, 24.2 and 40.9%, respectively). The differences in wastewater toxicity reduction were associated with wastewater feeding frequency.

Based on the toxicity classification of wastewater (Table 4), a reduction in toxicity class from high (for influent) to average (for effluent) can be observed and was validated by statistical analyses (Mann–Whitney U test, $p < 0.05$, Table 4).

**Table 4.** The TU value and toxicity classification of wastewater from CWs.

| Types of Columns       | $A. fischeri$ | $D. magna$, 24 h | $D. magna$, 48 h | $L. minor$ |
|------------------------|-------------|-----------------|-----------------|-----------|
| Influent CTRL          | 1.1 ± 0.3 a | 3.1 ± 0.8 b     | 4.1 ± 0.9 b     | 11.2 ± 1.7 a |
| Influent PhC           | 1.0 ± 0.3 a | 2.8 ± 1.0 b     | 3.9 ± 0.8 b     | 11.5 ± 2.1 a |
| R1-CTRL-Planted       | 1.3 ± 0.1 a | 2.7 ± 1.5       | 3.6 ± 1.4       | 8.8 ± 2.9 |
| R1-PhC-Planted        | 1.4 ± 0.1 a | 2.3 ± 0.8       | 3.6 ± 1.4       | 7.1 ± 1.7 a |
| R1-CTRL-Unplanted      | 1.3 ± 0.1 a | 1.6 ± 0.6 b     | 2.8 ± 1.3 b     | 6.3 ± 3.5 |
| R1-PhC-Unplanted       | 1.0 ± 0.1 a | 1.7 ± 0.4 b     | 2.3 ± 0.6 b     | 7.4 ± 3.7 |
| R2-CTRL-Planted       | 0.5 ± 0.1 a | 0.9 ± 0.8 b     | 2.7 ± 2.0 b     | 4.7 ± 3.6 a |
| R2-PhC-Planted        | 0.4 ± 0.2 a | 0.5 ± 0.8 b     | 1.2 ± 0.9 b     | 3.1 ± 2.4 a |
| R2-CTRL-Unplanted      | 0.9 ± 0.3 a | 1.6 ± 0.4 b     | 2.4 ± 0.8 b     | 4.0 ± 1.6 a |
| R2-PhC-Unplanted       | 0.6 ± 0.1 a | 0.6 ± 0.5 b     | 1.5 ± 0.8 b     | 2.2 ± 1.3 a |

To determine the hazardous concentration of wastewater for 5% of species, $HC_5$ values were calculated using a species sensitivity distribution model [44,45]. The $HC_5$ values and 95% confidence limits, including lower limit (LL) and upper limit (UL), are shown in Table 5. All samples met the normal distribution criteria for the Kolmogorov–Smirnov (K-S) test ($\alpha = 0.025$). The analyses represent $n = 16$ repeats, and the critical value of K-S statistics was 0.995. The NOEC parameter was used as a baseline to calculate $HC_5$, denoting the highest concentration with no observed harmful effect on model organisms. Low $HC_5$ values indicate that contamination of surface water with the analyzed wastewater can cause harmful effects to organisms living in this environment. Even slight contamination of the environment with raw wastewater possessing a low $HC_5$ value can be dangerous for living organisms (determined $HC_5$ at the level of 5.7%). Treated wastewater demonstrated less harmful ecotoxicological properties, with $HC_5$ values for the effluents ranging from 15.8–24.0%.
Table 5. HC₅ values for wastewater from CWs.

| Types of Columns          | Parameter, % |
|---------------------------|--------------|
|                           | LL ¹         | HC₅          | UL ²         |
| Influent CTRL             | 4.0          | 5.7          | 7.2          |
| Influent PhC              | 4.0          | 5.8          | 7.3          |
| R1-CTRL-Planted          | 13.6         | 15.8         | 17.8         |
| R1-PhC-Planted           | 14.0         | 16.4         | 18.5         |
| R1-CTRL-Unplanted        | 13.9         | 17.2         | 20.7         |
| R1-PhC-Unplanted         | 18.6         | 22.1         | 25.1         |
| R2-CTRL-Planted          | 13.9         | 17.0         | 19.5         |
| R2-PhC-Planted           | 16.0         | 18.5         | 20.8         |
| R2-CTRL-Unplanted        | 18.1         | 21.5         | 23.1         |
| R2-PhC-Unplanted         | 19.4         | 24.0         | 26.5         |

¹ Lower limit; ² upper limit.

4. Discussion

4.1. Chemical Parameters of Treatment Process

The results showed that the effect of the feeding frequency affected all determined chemical parameters. In the case of TOC concentration, higher removal rates were observed for R2 columns (feeding regime of 5 times a week in a volume of 1 L). Similar relationships were reported by Saeed and Sun [46], who showed that the dosing regime and the type of CWs used can affect the removal efficiency of organic compounds. These findings are reflected in the literature concerning vertical-flow CWs [47,48]. The mode of CW operation can impact the oxidation and reduction processes occurring in the system and the diffusion of oxygen into the bed. Periodic dosing that provides oxygen conditions can result in better removal efficiency than continuous feeding [49]. However, the denitrification process can be limited in such systems [50,51]. For planted columns, improved N-NH₄ removal from wastewater may be related to uptake by plants and stimulation of rhizosphere bacteria rhizosphere to oxidize ammonium nitrogen [52].

The presence of plants in CWs may therefore play a role in the removal of some pharmaceuticals, such as DCF. Plants can take up contaminants but also stimulate development of bacteria in the rhizosphere [49]. In addition, the presence of plants can affect the pharmaceuticals’ removal through root exudates. The release of some plants’ metabolites, such as threonine, citronellic acid or acrylic acid derivate, was combined to higher PhC removal in the columns with Phragmites australis plants [53].

The uptake of xenobiotics by plants is an important process contributing to the removal of micropollutants. This process mainly depends on the sorption of pollutants onto soil particles, as the primary substrate of the CW system. Only contaminants that are in the pore water can be taken up by the root system. Sorption also affects the availability of PPCPs to microorganisms, and consequently, the effectiveness of microbial degradation [54]. Diffusive translocation of pharmaceuticals into plant tissues depends mainly on the physicochemical properties of these compounds. The most important parameters are water solubility and hydrophobicity, characterized in terms of the octanol:water partition coefficient, log Kₐw, of the compound [55]. Moderate hydrophobicity, as indicated by a log Kₐw in the range of 0.5–3.5, is considered optimal for a compound to be taken up by plants [56]. Chemical compounds with a log Kₐw above 3.5 (such as DCF, Appendix A—Table A1) are less likely to diffuse into the roots of plants; for this reason, the bacteria from the rhizosphere are required to be more involved in DCF removal [55,56]. PPCPs can be removed by plants in the CW directly, as described above, or indirectly. The indirect effect of plants in removing xenobiotics is mainly to promote the growth and activity of rhizosphere microorganisms through the plant root system. In addition, each plant species
used in CW has a specific population of microorganisms in the rhizosphere, and thus the
distribution of pollutants, including PPCPs, may differ [57]. In our study, the presence of
M. giganteus in CWs was not significant for the removal of pharmaceuticals, but in earlier
work we showed a positive effect of another plant, Phragmites australis, on the removal of
pharmaceuticals [30].

The designed technological parameters also have a significant impact on the efficiency
of wastewater treatment in CWs. For submerged wetlands, periodic aeration of the bed
is recommended to ensure aerobic conditions, providing better removal of carbon and
nitrogen compounds. In addition, the use of plants helps transfer oxygen to the root portion
of plants located in CW beds [58]. The composition of the bed, which is involved in the
filtration or absorption of contaminants, among other things, is also an important element
affecting the efficiency of CWs [59].

For full-scale CWs, the climatic variable will also affect the efficiency of contaminant
removal. In the case of our laboratory-scale CWs, this impact has been minimized by
controlling basic parameters (such as temperature, light intensity, length of day and night).
However, the impact of these parameters on CW performance should be investigated and
requires further validation. Comparing the results of DCF and SMX removal in CWs with
those reported by other researchers, it can be concluded that the results we obtained are
average for biological systems. The biodegradation process leads to only moderate removal
of active pharmaceutical substances [30,60–62].

In the literature, one can find information about unconventional studies related to the
removal of SMX from wastewater. For example, the degradation of SMX by microalgae was
studied [63]. The observed maximum biodegradation of SMX was 99.3% and was achieved
by a pure culture of Chlorella pyrenoidosa with initial SMX concentration of 0.1 mg L\(^{-1}\) [64].
In contrast, the removal efficiency of SMX in the bacterial–microalgae hybrid system
was lower, with the maximum elimination reaching 40.84 ± 6.0% when the initial SMX
concentration was 1 mg L\(^{-1}\) [63]. Thus, in our study, pharmaceutical removal was at a
satisfactory level, especially considering the low operating costs.

4.2. Activity of M. giganteus Enzymes

An important factor impacting the use of plants in CWs is potential toxicity of the
wastewater. Oxidative stress, a state of imbalance between the production of reactive
oxygen species (ROS) and their elimination by antioxidant systems, is one of the measures
of harmfulness of wastewater to organisms. Many substances present in the environment
can cause increased ROS production and resultant oxidative stress, leading to negative
effects on living organisms [65]. Factors that can affect oxidative stress include temperature,
oxxygenation, salinity, metal ions, pesticides and other chemical contaminants [65]. In a
healthy organism, there should be a balance between ROS production and the protective
antioxidant systems and organisms have developed a series of defense mechanisms against
ROS. These mechanisms include prevention of excessive ROS formation, termination of
radical reactions and removal of the effects of ROS by means of antioxidant enzymes that
are designed to remove excess reactive molecules [66].

The antioxidant enzymes CAT and SOD are responsible for reducing oxidative stress
and play a very important role in plant survival [67]. In plant cells, SOD can be divided into
three groups: Fe SOD (located in chloroplasts), Mn SOD (in mitochondria and peroxisomes)
and Cu-Zn SOD (located in chloroplasts and cytosol). The action of SOD represents the
most important defense mechanism in plants and crucial for the detoxification process [68].

The activity of antioxidant enzymes is often used to gauge the effect that impurities
have on plants used in CWs [47,68,69]. Elevated CAT and SOD activity may result from
extended contact of M. giganteus with the wastewater being analyzed, since the leaves
used for enzymatic assays were collected on 241–290 days after first exposure of plants to
wastewater. Yan et al. [70] similarly suggested that an increase in antioxidant activity is
associated with an increase in the contact time of plants with wastewater and also showed
that plants can take up and potentially metabolize pharmaceuticals.
Catalase is considered to be a bioindicator of oxidative stress from various pollutants. In *Oriza sativa* plants, an increase in the activity of this enzyme was observed under the influence of wastewater from a rice mill in pot culture [71]. In addition, in the case of plant contact with heavy metals, a relationship was observed between the concentration of the tested substances and the activity of CAT and SOD. The increase in catalase activity was correlated with the concentration of heavy metals in the *Typha latifolia* leaves, but the rhizomes of the same plants did not show increased catalase activity [72]. In our study, we observed an increase in CAT and SOD activity in *M. giganteus* leaf samples taken from racks fed with wastewater containing DCF and SMX. We observed an increase in CAT activity of 120% for R1 columns and 121% for R2 columns with respect to the enzyme activity in *M. giganteus* leaves watered with control (synthetic wastewater). As for SOD activity, an increase of 141% and 129% was observed for Rack 1 and Rack 2, respectively.

The activity of antioxidant enzymes is a very useful tool for assessing the long-term harmfulness of tested substances to aquatic plants. An increase in enzyme activity indicates the onset of oxidative stress, and therefore a disturbance of homeostasis in the plant. By assessing enzyme activity, we can detect changes before visible damage occurs (e.g., wilting of leaves, stunting of plant growth). It would be a very valuable study to determine the activity of antioxidant enzymes at multiple time points during the experiment. This would give a picture of the changes that occur in plants during prolonged exposure to contaminants.

4.3. Toxicity of Wastewater

The operating parameters of the CWs affected the treated wastewater and thereby the effectiveness of treatment process. However, the removal of organic carbon, nitrogen compounds or xenobiotics cannot be treated as synonymous with wastewater toxicity reduction.

In the case of the *A. fischeri*, a reduction in the toxicity of wastewater was observed after the treatment by all types of columns with lower feeding frequency (R2) and by R1-PhC-Unplanted columns. An increase in toxicity observed in wastewater from the R1 columns (feeding frequency of twice per week at a volume of 2.5 L) might be associated with the generation of more toxic decomposition products [73] or slower distribution of primary metabolites. Sulfonamide antibiotics have been shown to undergo transformation in aqueous environments by biodegradation, photolysis or hydrolysis. Majewsky and coauthors [74] found that some SMX transformation products, especially 4-hydroxy-SMX and N4-hydroxy-acetyl-SMX, exhibit antimicrobial activity. The antimicrobial activity was tested for luminescence inhibition on *Vibrio fischeri*. It seems that in our study we observed exactly the same phenomenon of persistence of antimicrobial activity of SMX derivatives. This is also confirmed by our previous studies [30], in which we demonstrated the presence of SMX degradation products in CW effluents; these were the products of (mono-, di-, tri-)hydroxylation, demethylation and deamination reactions. The above examples indicate that even partially or completely transformed compounds (even if the parent compounds are not detectable in the environment) can cause similar or even greater effects. Such an effect is related to the pseudo-persistence of the antimicrobial agents [20].

It should also be kept in mind that wastewater is a cocktail of different substances that undergoes transformations during treatment that result in the formation of new compounds with different properties. Usually, the treatment process also results in a reduction in possible toxicity, but this is not always the case. For example, Punzi et al. [75] observed that textile wastewater showed higher toxicity to *V. fischeri* after anaerobic treatment. The introduction of an ozonation process significantly reduced the harmfulness of wastewater, indicating that the availability of oxygen may be a key element in the removal of contaminants from wastewater [75]. Our results indicate that the frequency of dosing of all wastewater samples affected toxicity to *A. fischeri* and *L. minor*. In the case of *D. magna*, the method of feeding affected toxicity only in wastewater samples containing pharmaceuticals. It was found that the toxicity of wastewater treated in R2 columns, dosed at 5 doses per week in a volume of 1 L per dose, was lower than that of wastewater treated
in R1 columns: two doses per week in a volume of 2.5 L per dose. The literature on the frequency of wastewater dosing and its effect on wastewater toxicity is sparse. Previously, it was observed that the frequency of wastewater dosing had a significant effect on the removal of DCF and SMX in the same experimental system, but operating under different experimental conditions [30]. The toxicity of wastewater to V. fischeri was also partially influenced by the feeding regime used. Ávila et al. [50] analyzed the effect of HLR on leachate toxicity in hybrid artificial CWs. The experimental system was operated at HLRs of 0.06, 0.13 and 0.18 m$^3$ m$^{-2}$ d$^{-1}$. The authors showed that in continuous feeding mode, high HLRs were able to reduce overall effluent toxicity by 90%. The presence of plants was shown to affect wastewater toxicity only toward A. fischeri and D. magna. A. fischeri luminescence was inhibited only by wastewater from R1-PhC and R2-CTRL columns (Figure 4A). The D. magna immobilization assay (Figure 4B,C) yielded statistically significant differences in toxicity between wastewater from planted and unplanted columns after 48 hrs of incubation with effluent from R1-PhC columns (Student’s t-test, $\alpha = 0.05$). The presence of M. giganteus plants in tested columns did not have a statistically significant effect on L. minor growth inhibition (Figure 4D). It had been previously observed that the toxicity of wastewater was 5–10% lower in columns that were not planted with Phalaris arundinacea [30]. This observation was associated with the dissolved organic carbon concentration. It has also been suggested that plant excretions may affect the toxicity of effluents from CWs. Root systems release various organic substances such as anaerobic metabolites, organic acids, steroids and even antimicrobial compounds. In addition, they may also secrete phytosiderophores, compounds that chelate iron, zinc, copper and manganese ions, which can affect the wastewater treatment process [76].

These findings suggest that DCF and SMX contents in wastewater are not the primary determinants of toxicity toward bioindicators as have been suggested by preliminary tests [77]. A statistically significant effect of pharmaceutical content on toxicity towards A. fischeri (Student’s t-test, $\alpha = 0.05$) was observed only in wastewater from unplanted columns. Toxicity of pharmaceutical-containing wastewater towards L. minor was observed only with regard to wastewater from R2-Unplanted columns, and toward D. magna (after 48 hrs of incubation) by wastewater from R1-Planted and R2-Unplanted columns.

The hazardous concentration for 5% of species was calculated (Table 5) to assess the risks that raw and treated wastewater may enter into the environment. The HC$_5$ value means the concentration of the substance, in this case wastewater, which is safe for 95% of species [44]. It is a worldwide problem that treated effluents constitute a considerable fraction of water in receiving waters under drought conditions as an effect of the climate change [78,79]. For this reason, the use of appropriate dilution factors can be crucial for environmental protection. Moreover, environmental samples are a mixture of different materials, and their exact composition is unknown and changes over time. For this reason, determining the harmful concentration value toward 5% of species may be valuable in assessing the purification process, even in the absence of complete information regarding wastewater composition.

In summary, wastewater from R2 columns was less harmful to aquatic bioindicators than the effluent from R1 columns. Toxicity tests showed that the frequency of dosing had a significant impact on the ecotoxicological properties of wastewater. The predominance of aerobic processes in columns with lower feeding frequency (R2) (wastewater dispensed 5 times a week in a volume of 1 L) provides better conditions for removing organic compounds, N-NH$_4$ and pharmaceuticals, and resulted in superior overall detoxification of effluents.

It is also worth mentioning that the influence of TOC and N-NH$_4$ on the toxicity of wastewater was observed. It was found that there was correlation between TOC and the toxicity of samples. It was also observed that higher correlation coefficients were obtained when results were divided into two groups: samples with TOC < 100 mg L$^{-1}$ (which corresponds to treated wastewater) and samples with TOC $\geq$ 100 mg L$^{-1}$ (raw wastewater). For each bioindicator, the correlation between toxicity, TOC and N-NH$_4$
(investigated as related parameters) was determined, while also identifying parameters for which wastewater was nontoxic to organisms. Appendix A—Figure A4 provides an example of the correlations between the analyzed parameters and wastewater toxicity toward the bioindicator D. magna. The calculated correlations presented in Appendix A—Figure A4 are relevant to wastewater samples that comprise the general properties of household wastewaters and do not contain additional specific toxic compounds aside from pharmaceuticals.

The equations obtained from correlation analysis between the chemical parameters of wastewater and their toxicity are presented in Appendix A—Table A7. According to the Persoone et al. [41] classification, the TU value for wastewater below 0.4 is regarded as safe for the environment. Since TOC and N-NH$_4$ tolerance have been analyzed in combination, if the concentration of organic compounds in the sample is very low (near zero), then model organisms may tolerate higher concentrations of N-NH$_4$ (up to 26 mg L$^{-1}$) while maintaining the same TU toxicity level of 0.4. When the N-NH$_4$ concentration tends to zero, the bioindicators’ tolerance to TOC concentration is approximately 35 mg L$^{-1}$. When TOC is above 100 mg L$^{-1}$ (raw wastewater), wastewater toxicity is very high (exhibiting a mean TU > 2) even if N-NH$_4$ concentration tends to zero. This influence of primary contaminants, expressed as TOC and N-NH$_4$ concentration, on the ecotoxicological properties of wastewater is an important observation with relevance to future long-term experiments involving wastewater of known composition.

Detoxification, according to the definition proposed by Fortney et al. [80], is a set of processes whose purpose is to identify, neutralize and eliminate toxic substances and their degradation products. This definition can refer to both the medical and environmental aspects of this process. Therefore, detoxification can be defined as a process of reducing the toxicity of a substance or wastewater to an environmentally safe level. According to the above definition, detoxification of wastewater was not achieved during the present study, as all effluent samples from CWs exhibited average toxicity to aquatic bioindicator organisms; however, it was confirmed that ecotoxicity testing of wastewater provides a broader and deeper insight to the efficacy of wastewater treatment processes. Ecotoxicological analysis of wastewater is not required in the majority of European countries, where the assessment of wastewater treatment is typically limited to the analysis of physicochemical parameters. Treated wastewater must be characterized by an appropriate level of contaminants or a sufficiently high removal efficiency of a given parameter before being introduced into the natural environment [17]. The data presented here emphasize that supplementing physicochemical analyses with ecotoxicological tests is very valuable for improved environmental protection.

5. Conclusions

The conducted research showed that the use of bioindication methods complements the information obtained from standard physicochemical analysis. Only both types of results give a full picture of the threat posed by the analyzed pollutants to the natural environment. The ecotoxicological analysis provides information on the harmfulness of pollutants in the environment. The physicochemical analysis allows for only the assessment of the effectiveness of the wastewater treatment process; however, the by-products generated during the purification process may often show higher biological activity than the parent product. Therefore, discharge of treated wastewater into the environment might pose a serious risk to living organisms. The use of both analyses allows for a complete evaluation of the treated wastewater properties.

The reduction in wastewater toxicity was observed in the laboratory model of the vertical-flow CWs; however, detoxification of wastewater was not achieved, as confirmed by the HC$_5$ values. Raw wastewater was characterized as highly toxic, while treated wastewater was classified as exhibiting average toxicity toward aquatic organisms. More frequent feeding resulted in improved ecotoxicological properties of wastewater effluents, which was assumed to be due to greater bed oxygenation. The presence of M. giganteus
plants was not a determining factor of the efficacy of the purification process. However, it was observed for the first time that the antioxidant enzyme activity detected in *M. giganteus* leaves was influenced by the operating parameters of the CWs (wastewater dosing and the presence of pharmaceuticals).

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**Appendix A**

**Appendix A.1. Materials and Methods**

**Appendix A.1.1. Tested Pharmaceuticals**

| Compound | DCF | SMX |
|----------|-----|-----|
| CAS number | 15307-86-5 | 723-46-6 |
| Molecular formula | \(C_{14}H_{11}Cl_2NO_2\) | \(C_{10}H_{11}N_3O_3S\) |
| Molar mass, g mol\(^{-1}\) | 296.15 | 253.28 |
| \(pK_a\) | 4.15 | 5.6–5.7 |
| \(\log K_{OW}\) | 4.51 | 0.89 |
| Maximal concentration in surface water | 18.74 | 11.92 |
| Maximal concentration in groundwater | 0.59 | 1.11 |
| Maximal concentration in wastewater effluent | 5.5 | 6.0 |

\(^1[69] \quad ^2[70] \quad ^3[71] \quad ^4[72] \quad ^5[73].\)

**Appendix A.1.2. Experimental System of CWs**

| Chemical Compounds | Concentration, mg L\(^{-1}\) | Food Ingredients | Concentration, mg L\(^{-1}\) | Trace Metals | Concentration, mg L\(^{-1}\) |
|--------------------|-----------------------------|------------------|-----------------------------|--------------|-----------------------------|
| CH\(_3\)COONa       | 510.4                       | yeast extract    | 264                         | KCr(SO\(_4\))\(_2\)·12H\(_2\)O | 0.96           |
| urea               | 208.76                      | skim milk powder | 118                         | CuSO\(_4\)·5H\(_2\)O    | 0.781          |
| KH\(_2\)PO\(_4\)    | 41.37                       |                  |                             | NiSO\(_4\)·7H\(_2\)O    | 0.359          |
| peptone            | 40                          |                  |                             | ZnCl\(_2\)            | 0.208          |
| FeSO\(_4\)·7H\(_2\)O| 11.6                        |                  |                             | MnSO\(_4\)·H\(_2\)O    | 0.108          |
| MgSO\(_4\)·7H\(_2\)O| 4.408                       |                  |                             | PbCl\(_2\)            | 0.1            |

All reagents were purchased from Avantor (Gliwice, Poland). The prepared synthetic wastewater was dosed to the surface of the bed, and then flowed vertically downwards until it reached the outlet zone.
Appendix A.1.3. Chemical Analysis of Wastewater

The wastewater samples (obtained by 8 sample collections) were filtered prior to further analysis. N-NH$_4$ concentration was analyzed by means of an Ammonium Test kit (test no. 1.00683.0001, Merck, Germany). TOC was determined by TOC-L analyzer (Shimadzu Corporation, Kyoto, Japan).

Pharmaceuticals concentrations were monitored by HPLC using a C18 Hypersil™ Gold column (250 mm × 4.6 mm, pore size: 5 µm; Thermo Scientific, Warsaw, Poland). The mobile phase consisted of a mixture of acetonitrile and acetate buffer (pH 5.7) in a volume ratio of 40:60 and was applied at an isocratic flow rate of 1.0 mL min$^{-1}$. The retention time for DCF was 8.4 ± 0.3 min, and for SMX was 6.4 ± 0.2 min. The limit of quantification was 0.2 mg L$^{-1}$. The tests were performed at four wavelengths: 220, 240, 268 and 280 nm. Data were analyzed using Chromeleon™ v. 6.8 software (Dionex Corporation, Sunnyvale, CA, USA).

Appendix A.1.4. Activity of Antioxidant Enzymes in $M$. giganteus

CAT activity (EC 1.11.1.6) was determined using a static method, measuring decomposition of hydrogen peroxide at a single time point, as described by [33]. The reaction was stopped after 4 min by adding 32.4 mM ammonium molybdate, which reacts with undecomposed H$_2$O$_2$ to form a colorimetric complex the intensity of which can be measured spectrophotometrically at 405 nm. A 65 mM solution of H$_2$O$_2$ in 0.06 M sodium phosphate buffer (pH 7.4) was used as the substrate. Enzyme activity is expressed in terms of µmol H$_2$O$_2$ min$^{-1}$ mg$^{-1}$ protein.

SOD activity (EC 1.15.1.1) was measured using the self-oxidation reaction of adrenaline to adrenochrome at pH 10.2 as described by [34]. The measure of SOD activity is the inhibition of adrenaline oxidation over 4 min reaction time, using a 10 mM solution of adrenaline in 0.01 M HCl as reaction substrate. The amount of oxidized adrenaline is measured spectrophotometrically at 480 nm. SOD activity is expressed in terms of U min$^{-1}$ mg$^{-1}$ protein. The arbitrary unit of activity (U) is defined as the amount of enzyme that leads to 50% inhibition of adrenaline oxidation within 1 min compared to auto-oxidation one would observe in a blank sample.

Appendix A.1.5. Toxicity Tests towards Aquatic Organisms

Table A3. Composition of synthetic sea water used as a control solutions in $A$. fischeri toxicity test.

| Component       | Concentration, g L$^{-1}$ |
|-----------------|---------------------------|
| NaCl            | 22.0                      |
| MgCl$_2$·6H$_2$O| 9.7                       |
| Na$_2$SO$_4$    | 3.7                       |
| CaCl$_2$        | 1.0                       |
| KCl             | 0.65                      |
| NaHCO$_3$       | 0.2                       |
| H$_3$BO$_3$     | 0.023                     |
Table A4. The classification of wastewater according to the TU value [42].

| TU Value | Toxicity Classification |
|----------|-------------------------|
| <0.4     | no toxicity             |
| 0.4–1    | low toxicity            |
| 1–10     | average toxicity        |
| 10–100   | high toxicity           |
| >100     | extreme toxicity        |

Appendix A.2. Results

Figure A1. TOC concentration (mg L$^{-1}$) of wastewater from CWs. (a) Statistically significant differences between the wastewater influent and effluent (Mann-Whitney U test, $p < 0.05$); (b) Statistically significant differences between counterpart types of columns (CTRL or PhC) with different frequencies of wastewater dosing (Mann-Whitney U test, $p < 0.05$); (c) Statistically significant differences between control wastewater and wastewater containing pharmaceuticals (Mann-Whitney U test, $p < 0.05$).
Figure A2. N-NH$_4$ concentration (mg L$^{-1}$) in wastewater from CWs. (a) Statistically significant differences between the wastewater influent and effluent (Mann-Whitney U test, $p < 0.05$); (b) Statistically significant differences between counterpart types of columns (CTRL or PhC) with different frequency of wastewater dosing (Mann-Whitney U test, $p < 0.05$); (c) Statistically significant differences between control wastewater and wastewater containing pharmaceuticals (Mann-Whitney U test, $p < 0.05$).

According to Li et al. [55], pharmaceuticals removed via CWs can be classified as easily removable (removal rate $> 70\%$), moderately removable (50–70\%), not-easily removable (20–50\%) and almost non-removable (removal rate $< 20\%$). According to this classification, DCF may be classified as a moderately removable compound (on average 61.5\% removal), whereas SMX was easily removable by CWs treatment (on average 88.4\% removal).

Table A5. Activity of antioxidant enzymes in M. giganteus.

| Types of Columns    | Activity of Antioxidant Enzymes |
|---------------------|---------------------------------|
|                     | CAT $\mu$mol H$_2$O$_2$ min$^{-1}$ mg$^{-1}$ Protein $^{-1}$ | SOD $\mu$mol U min$^{-1}$ mg$^{-1}$ Protein $^{-1}$ |
| R1-CTRL-Planted     | 136.7 ± 18.4                     | 37.2 ± 4.5               |
| R1-PhC-Planted      | 165.3 ± 15.4                     | 52.4 ± 8.2               |
| R2-CTRL-Planted     | 169.7 ± 13.4                     | 46.1 ± 6.9               |
| R2-PhC-Planted      | 205.9 ± 14.5                     | 59.6 ± 6.7               |
Figure A3. DCF (A) and SMX (B) concentration (mg L$^{-1}$) in wastewater from CWs. (a) Statistically significant differences between the wastewater influent and effluent (Mann-Whitney U test, $p < 0.05$); (b) Statistically significant differences between DCF and SMX concentration (Mann-Whitney U test, $p < 0.05$); (c) Statistically significant differences between counterpart types of columns (CTRL or PhC) with different frequency of wastewater dosing (Mann-Whitney U test, $p < 0.05$); (d) Statistically significant differences between wastewater from planted columns and from columns without plants (Mann-Whitney U test, $p < 0.05$).

Table A6. Wastewater toxicity reduction as a result of treatment in CWs.

| Types of Columns | The Wastewater Toxicity Reduction, % |
|------------------|--------------------------------------|
|                  | A. fischeri | D. magna, 24 h | D. magna, 48 h | L. minor |
| R1-CTRL-Planted  | −24.8       | 14.6           | 12.4           | 29.1     |
| R1-PhC-Planted   | −42.2       | 19.4           | 8.9            | 38.7     |
| R1-CTRL-Unplanted| −22.5       | 48.9           | 32.9           | 54.2     |
| R1-PhC-Unplanted | 0.3         | 40.8           | 42.5           | 41.5     |
| R2-CTRL-Planted  | 55.3        | 72.6           | 34.2           | 69.0     |
| R2-PhC-Planted   | 62.1        | 82.6           | 70.1           | 78.3     |
| R2-CTRL-Unplanted| 14.3        | 49.2           | 41.4           | 65.4     |
| R2-PhC-Unplanted | 43.3        | 77.3           | 62.4           | 82.8     |
**Figure A4.** Correlations analysis between toxicity to *D. magna* and the chemical parameters of wastewater. (A) Wastewater with TOC < 100 mg L\(^{-1}\); (B) Wastewater with TOC ≥ 100 mg L\(^{-1}\); where x is the TOC concentration (mg L\(^{-1}\)) and y is the N-NH\(_4\) concentration (mg L\(^{-1}\)).

**Table A7.** Equations obtained from correlation analysis.

| Test Organisms | TOC Concentration, mg L\(^{-1}\) |
|----------------|----------------------------------|
|                | <100 mg L\(^{-1}\)              | ≥100 mg L\(^{-1}\)             |
| *A. fischeri*  | TU = -0.5706 + 0.0216\(x\) + 0.0213\(y\) | TU = 1.6187 + 0.0037\(x\) + 0.0037\(y\) |
| *D. magna*, 24 h | TU = -0.5706 + 0.0216\(x\) + 0.0213\(y\) | TU = 1.6187 + 0.0037\(x\) + 0.0037\(y\) |
| *D. magna*, 48 h | TU = -0.1875 + 0.0334\(x\) + 0.0266\(y\) | TU = 2.0676 + 0.0044\(x\) + 0.0075\(y\) |
| *L. minor*     | TU = -1.4193 + 0.045x + 0.0803y | TU = 8.9237 + 0.01x − 0.0031y |

Where: x is the TOC concentration (mg L\(^{-1}\)), while y is the N-NH\(_4\) concentration (mg L\(^{-1}\)).

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