Low-cost approaches for the removal of terbuthylazine from agricultural wastewater: Constructed wetlands and biopurification system

Georgios D. Gikas\textsuperscript{a}, Marta Pérez-Villanueva\textsuperscript{b}, Mathaios Tsioras\textsuperscript{a}, Christos Alexoudis\textsuperscript{c}, Greivin Pérez-Rojas\textsuperscript{b}, Mario Masís-Mora\textsuperscript{b}, Verónica Lizano-Fallas\textsuperscript{b}, Carlos E. Rodríguez-Rodríguez\textsuperscript{b}, Zisis Vryzas\textsuperscript{c}, Vassilios A. Tsihrintzis\textsuperscript{d}\textsuperscript{,}\textsuperscript{e}

\textsuperscript{a} Laboratory of Ecological Engineering and Technology, Department of Environmental Engineering, School of Engineering, Democritus University of Thrace, 67100 Xanthi, Greece

\textsuperscript{b} Centro de Investigación en Contaminación Ambiental (CICA), Universidad de Costa Rica, 2060 San José, Costa Rica

\textsuperscript{c} Laboratory of Agricultural Pharmacology and Ecotoxicology, Department of Agricultural Development, Democritus University of Thrace, 68200 Orestias, Greece

\textsuperscript{d} Centre for the Assessment of Natural Hazards and Proactive Planning & Laboratory of Reclamation Works and Water Resources Management, Department of Infrastructure and Rural Development, School of Rural and Surveying Engineering, National Technical University of Athens, Zografou 15780 Athens, Greece

\textsuperscript{⁎} Corresponding author.

\textit{E-mail addresses:} ggkikas@env.duth.gr (G.D. Gikas), carlos.rodriguezrodriguez@ucr.ac.cr (C.E. Rodríguez-Rodríguez), zvryzas@agro.duth.gr (Z. Vryzas), tsihrin@otenet.gr, tsihrin@survey.ntua.gr (V.A. Tsihrintzis).

https://doi.org/10.1016/j.cej.2017.11.031

Received 8 September 2017; Received in revised form 4 November 2017; Accepted 4 November 2017

Available online 06 November 2017

Chemical Engineering Journal 335 (2018) 647–656

\textcopyright 2017 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

GRAPHICAL ABSTRACT

ARTICLE INFO

Keywords:
 Constructed wetland
 Biopurification system
 Terbuthylazine
 Bioremediation
 Phytoremediation

ABSTRACT

Constructed wetlands (CWs) and biopurification systems (BPS) present two low-cost approaches for the removal of pesticides from waters of agricultural origin. Both strategies were tested in the treatment of the herbicide terbuthylazine, a triazine of worldwide use. Three horizontal subsurface flow (HSF) CW systems were operated continuously for one year; the planted CWs (containing either Phragmites australis or Typha latifolia) were able to remove up to 73.7% and 58.4% of the pesticide, respectively, and exhibited a markedly superior performance compared to the unplanted CW. However, by the end of the treatment period, some symptoms of phytotoxicity were observed in the plants of the CWs, which are related to high terbuthylazine concentrations in plant material. A coconut fiber-containing biomixture was used in BPS which was able to rapidly remove terbuthylazine, with an estimated half-life of 8.1 d, the fastest so far reported in these systems. However, the biomixture failed to detoxify the matrix, according to ecotoxicological tests of seed germination. The current data suggests that...
coupled/hybrid configurations comprising CWs and BPS in series could provide increased effectiveness and a low-cost technology to remove terbutylazine from highly contaminated water.

1. Introduction

Pesticides are widely used all over the world as they are necessary to sustain the agricultural sector; in particular, the use of herbicides is a major component in all integrated pest management systems. Triazines comprise one of the most widely used families of pesticides for the control of grass and broad-leaved weeds in a variety of crops, like wheat, grape, peaches, sorghum, asparagus, corn, barley, apple, banana, citrus, pineapple, sugarcane, coffee and maize, as well as for non-agricultural purposes including forestry and maintenance of roads [1,2]. Due to the prohibition of atrazine in the European Union, terbutylazine was gradually employed as the replacing herbicide [3], which is considered as the most persistent triazine herbicide in surface environments [4]. Its presence has been reported worldwide in water bodies [5,6] and is among the most frequently detected pesticides in surface and groundwater in Greece [7–10].

Due to the use of pesticides, ecosystems suffer from negative impacts, such as soil and air, and surface and groundwater contamination when these chemicals enter the environment by diffuse or point-source pollution [11,12]. Mixing and loading of pesticides, filling and washing

Fig. 1. (a) Schematic design of the horizontal subsurface flow constructed wetland pilot-scale units; (b) view of the three pilot-scale HSF-CWs; (c) preparation of bio-mixture and pilot biopurification system.
of spray equipment, equipment leaks, improper handling of tank mix left-overs, and pesticide waste disposal operations are activities that contribute to point-source pollution [13,14]. The application of good agricultural practices in the field results in a decrease of these pollution sources; nonetheless, the use of natural treatment approaches such as biopurification; systems and constructed wetlands may provide additional alternative means for pesticide elimination in an ecofriendly manner [13–16]. As a part of their remediation potential, these systems use the ability of some microbial populations to degrade complex chemical substances like pesticides, integrating them as part of metabolic pathways or for cometabolism processes [3,17].

Constructed wetlands (CW) constitute an effective, practical and low-cost option in treating runoff and various wastewaters, such as municipal, agricultural and industrial [18–22]. CWs mimic natural self-cleansing processes in a phytoremediation-mediated approach. Systems comprise basins with vegetation, where plants with specific known ability to accumulate or remove pollutants are used and represent one of the main components of CWs [23–25]. Pesticide interaction with soil, plant metaorganisms (including symbiotic, epiphytic and endophytic microbiome) and root exudates at the rhizosphere, specify their behavior within the plant (i.e., uptake, translocation, action, detoxification and excretion), and therefore, determine the efficiency of the CW [26]. Generally, pesticide removal occurs through physical, chemical and biological processes, which include retention, settling, plant uptake, adsorption and microbial breakdown, among others [16,27–29]. Among the research reports on CWs, only a small percentage have dealt with the fate of pesticides [30]. Limited previous studies have demonstrated the ability of wetlands in treating wastewaters polluted with triazines [31,32].

Biopurification systems (BPS) have been used for the removal of pesticides contained in effluents from agricultural activities. BPS are composed of three layers, i.e., grass, biomass mixture and clay, with biomass mixture being the main component of these systems [17]. The biomass mixture provides the microbial populations in charge of most degradation in the BPS, and typically contains: soil with pre-exposure history to the specific pesticide, which assures a microbial community adapted to the substance [33]; a lignocellulosic substrate, which promotes the colonization and activity of ligninolytic fungi, capable to degrade the pesticides [34]; and a humic-rich material to increase the adsorption of pesticides [12]. Moreover, some reports describe the elimination of herbicides, and particularly triazines, in biomass mixtures [35–38].

The purpose of this study was to examine the removal of terbuthylazine originating from point sources in the agricultural environment (e.g., spraying equipment rinsing sites) in two different low-cost bioremediation systems: a previously optimized biomass used in BPS and mature pilot-scale CWs. The biomass was optimized for pesticide removal and contains easily available substrates. On the other hand, the CW has been in operation since 2003 for the removal of several pollutants, including fungicides, herbicides, EDCs etc. [14,21,22]. Ecotoxicity and phytotoxicity of remaining terbuthylazine on D. magna and L. sativa were also assessed. A proposal of using the two systems in a sequence of remediation techniques is also discussed.

2. Materials and methods

2.1. Chemicals and reagents

Analytical standard of terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4-diamine, 98.5%) was acquired from Dr. Ehrenstorfer (Augsburg, Germany). Commercial formulation of terbuthylazine (Mumetal 50 SC and Terbusol 50 SC®, 50% w/v) was purchased from the market. Solvents and internal standard used are listed in Ruiz-Hidalgo et al. [39].

2.2. Experimental setup

2.2.1. Constructed wetlands

The experimental setup was located in the open-air space of the Laboratory of Ecological Engineering and Technology, Department of Environmental Engineering, Democritus University of Thrace (location: 41°08′47″ N, 24°55′09″ E). Three pilot-scale horizontal subsurface flow (HSF) CWs (named: MG-Z, MG-R and MG-C) were used (Fig. 1). The CWs were rectangular tanks (dimensions length × width × depth: 3 m × 0.75 m × 1 m; porous media thickness: 0.45 m). The three units were filled with the same porous media, medium gravel (MG, D50 = 24.4 mm, range 8–32 mm). Two were planted with different vegetation type (MG-C: cattail - Typha latifolia; MG-R: common reed - Phalaris arundinacea; MG-Z: reed - Schoenoplectus lacustris).

Fig. 2. Box-Whisker plots of the measured physicochemical parameters of the influent and effluents from the CW units MG-Z, MG-R, MG-C: (a) temperature; (b) pH; (c) electrical conductivity; (d) dissolved oxygen. The line inside the box (−) denotes median values, the upper box frame the 75th percentile, the lower box frame the 25th percentile and the dots (○) denote outliers.
Phragmites australis), while the MG-Z was kept unplanted and was used as control unit. The three CWs were established in 2003, and since then, they operated continuously treating wastewater [19,27,40]. Therefore, they are characterized as mature CWs. Detailed description of the design characteristics of these units is provided by Akratos and Tsihrintzis [40]. All CWs were loaded with water enriched with terbuthylazine at 0.4 mg/L on a daily basis from August 2014 to October 2015 by using a pump with a volume counter. Physicochemical properties of the influent and effluent of all CWs are presented in Fig. 2. The hydraulic residence times (HRT) that were applied were 6 and 8 days, which correspond to hydraulic loading rates (HLR) of 24 mm/d and 18 mm/d, respectively. The lower HRT (6 d) was applied during the warmer months, due to the wetland water requirements and the increased evapotranspiration, while in colder months, the higher HRT was used (8 d) [14,40,41]. The daily influent water volumes fortified with terbuthylazine were 54.0 L and 40.5 L for HRTs of 6 d and 8 d, respectively; they were divided in two equal doses which were introduced to the systems twice a day, at about every 12 h. Influent and effluent water samples were collected every 15 d from each CW for extraction and instrumental analysis in the laboratory. The appropriate terbuthylazine concentration for this experiment was decided by taking into consideration the application rates according to the formulation label. The concentration of terbuthylazine in the influent water to the CWs units was similar to the one found in the rinsing water of a 1000 L tank of a spraying equipment. The possible adverse effect of terbuthylazine concentrations on vegetation of the CWs were also taken into consideration. Thus, the CWs were loaded in the influent water with terbuthylazine (Table 1) at 0.4 mg/L. Physicochemical characteristics (Temperature, T; pH; electrical conductivity, EC; dissolved oxygen, DO) were measured in situ using WTW 197i (Wissenschaftlich Technische Werkstätten, Germany) series portable instruments. In December 2014 and September 2015, plants of both MG-R and MG-C systems were removed from the units and samples of roots, shoots and leaves were collected and analyzed to determine terbuthylazine concentrations.

2.2.2. Biopurification system

Coconut fiber, compost and soil at a volumetric composition of 45:13:42 was employed as biomixture for terbuthylazine removal and ecotoxicological assays (Fig. 1). Physical properties and chemical composition of the biomixture are listed in Huete-Soto et al. [36]. The biomixture was previously optimized for the removal of carbofuran and was designed taking into account several residues from the tropical areas of Costa Rica as newspaper paper, sugarcane bagasse, coconut fiber, rice husk and wood chips [42,43]. Triplicate trays (17 × 11 × 9.5 cm) were conducted containing approximately 700 g of the biomixture, in which a terbuthylazine solution was applied obtaining a concentration of 50 mg/kg; the herbicide concentration was chosen considering the application recommendations and an estimated volume of wastewater disposed in a 200 L BPS. The biomixture was manually mixed and then the trays were incubated at 25 °C until the end of the assay. Water content losses were frequently adjusted by manually mixed and then the trays were incubated at 25 °C until the end of the assay. Water content losses were frequently adjusted by manually mixing and then the trays were incubated at 25 °C until the end of the assay. Water content losses were frequently adjusted by manually mixing and then the trays were incubated at 25 °C until the end of the assay.

2.3. Analytical procedures

2.3.1. Terbuthylazine determination in different matrices

Water samples (200 mL) from the inlet and outlet of the CWs were first filtered (0.7 μm), and within 24 h the extraction procedure was performed by using solid-phase extraction in C18 (500 mg/6 mL) cartridges following the method described by Papaevangelou et al. [14]. The samples were subjected to HPLC-PDA analysis (Thermo Finnigan, Surveyor system equipped with LC solvent pump, degasser, auto-sampler and photodiode array detector). Chromatography was carried out on a Hypersil Gold 100 × 4.6, 5 μm HPLC column. The acquisition of the data was made at 215, 220 and 234 nm and quantification was conducted at 220 nm. The analytical method was validated according to the SANTE/11945/2015 requirements [44]. Terbuthylazine limit of detection and limit of quantification were 0.05 and 0.1 μg/L. Recoveries tested at 3 levels (400, 10 and 0.1 μg/L) ranged from 92 to 107%.

Terbuthylazine concentration on roots, shoots and leaves of Typha latifolia and Phragmites australis was determined by the modified “QuEChERS aceta” methodology, as described by Liu et al. [45]. Briefly, 10 mL of water was added to 2 g of plant material into a 50 mL tube, and was left to soak for 30 min. Then, 10 mL acetonitrile (0.1% acetic acid) was added and shaken for 1 min. Subsequently, 6 g anhydrous MnSO4 and 1.5 g sodium acetate were added and shaken. 1 mL of the upper phase (after centrifugation) was cleaned up by 50 mg PSA and 150 mg MgSO4. The mixture was vortexed for 3 min and centrifuged for 5 min. The supernatant was filtered (0.45 μm) and analyzed by HPLC-PDA as described above.

Extraction of terbuthylazine from biomixtures followed the method of Ruiz-Hidalgo et al. [39]. A mixture of water and acidified acetonitrile (formic acid 1% v/v) and a UPLC-MS/MS system (UPLC-1290 Infinity LC, triple quadrupole mass spectrometer model 6400, Agilent Technologies, CA) were used.

A Poroshell 120 EC-C18 column (100 mm × 2.1 mm i.d., 2.7 μm) at 40 °C was used for chromatographic separation. MS/MS operational conditions were as described in [43] and method validation data are shown in Table 2. Removal values for terbuthylazine were determined from triplicate systems per matrix, as percentages of the initial sample concentration.

2.3.2. Ecotoxicological assays

Elutriates from biomixtures samples, prepared according to the protocol EPA-823-B-01-002 [46], were used in the toxicity tests. The acute toxicity test of D. magna immobilization was performed using neonates, as described in the methodology EPA-821-R-02-012 [47]. EC50 was determined using the binomial method in TOXCALC – Toxicity Data Analysis Software (Tidepool Scientific Software, CA, USA). Toxicity results and their confidence limits at 95% were expressed as toxicity units (TU): TU = (EC50) − 1 × 100. The phytotoxicity of the biomixture during the experiment was monitored by triplicate seed germination tests with lettuce (L. sativa var. Georgia) [48]. Relative seed germination (SG), relative root elongation (RE) and germination index (GI) were calculated as described in our previous study [36].

2.4. Statistical analyses

The nonparametric Kruskal–Wallis test was used to determine

| Parameter | Terbutylazine |
|-----------|--------------|
| Chemical formula | C6H12ClN2 |
| Chemical group | Triazine |
| Chemical structure | |

Molecular weight (g/mol) 229.71
Solubility in water at 20 °C (mg/L) 6.6
Lipophilicity, Log Kow 3.4
Vapor pressure at 25 °C (mPa) 0.09
Adsorption coefficient Koc (mL/g) 231
Soil dissipation DT50 (d) 21.0
differences in mean removal efficiencies among the three HSF-CW units, because the majority of data failed to meet the assumption of normality and/or homogeneity. In cases where the difference between removal values was significant, the Mann–Whitney U test was used to evaluate pair comparisons. The statistical significant level was set at $p = .05$. Statistical analyses were performed using SPSS Statistics 17.0 for Windows.

3. Results and discussion

3.1. Removal in constructed wetlands

Fig. 2 presents box and whisker plots of the measured physicochemical parameters of the influent and effluents from the three CWs (i.e., MG-Z, MG-R and MG-C). The temperature values followed the seasonal variation in the three pilot-scale units and varied between 6.6 °C and 31.7 °C (Fig. 2a). Mean pH values of water in the three CWs were in the alkaline range (7.3 ± 0.3) without remarkable variations throughout the monitoring period (Fig. 2b). The EC values varied between 215 μS/cm and 979 μS/cm and the mean values were 399 μS/cm, 629 μS/cm, 588 μS/cm and 526 μS/cm for the MG-Z, MG-R, MG-C and influent, respectively (Fig. 2c). Higher EC values were measured during the warmer months when evapotranspiration rates were higher (May to August). The mean DO concentrations decreased from the influent of 7.1 ± 1.1 mg/L to the effluents of 2.9 ± 1.0, 2.9 ± 1.1, and 2.8 ± 0.9 mg/L for MG-Z, MG-R and MG-C, respectively (Fig. 2d).

Based on a previous research by Lv et al. [49], oxygen saturation plays a role in pesticides degradation in CWs during the winter period. Water temperature, pH values, evapotranspiration, electrical conductivity and hypoxic conditions are physicochemical properties that can affect the dissipation rate of terbuthylazine in the CW systems. Terbuthylazine is a week base with $pK_a = 2$ and its dissociation and movement within plants is directly related to pH values [2]. The Cl-triazines, like terbuthylazine, can be considered neutral herbicides in pH values of water samples (7.3 ± 0.3), while methyl- and methoxy-triazines are weak bases ($pK_a = 4.0–4.3$) [50,51]. Thus, sorption of terbuthylazine on the
CW support matrix is mainly dependent on the organic matter content, as lipophilicity is the most important property that regulates uptake of non-ionized herbicides. Moreover, its limited dissociation under pH values observed in water samples facilitates terbutylazine uptake by plant material. The optimum uptake by roots and translocation to shoots occurs for herbicides with Log Kow values 1–3 [52]. Terbutylazine, with a Log Kow of 3.4, could be translocated within the plant material and reached up to 73.7% removal efficiency [21]. The MG-R system was reported to have a low adsorption and bioavailability in the CWs. However, water physicochemical properties were similar in all effluent water. Thus, differences observed in terbutylazine removal among CWs tested cannot be attributed to the differential solubility, sorption and chemical dissipation in the water.

Fig. 3 shows the terbutylazine concentration (Fig. 3a) and removal rates (Fig. 3b) time series during the entire sampling period. Removal rates were calculated by Eq. (1):

\[
R = \frac{C_i - C_e}{C_i} \times 100
\]

where R is the removal rate (%), and C_i and C_e the influent and effluent terbutylazine concentrations (mg/L).

The mean (measured) influent concentration was 381 ± 61 μg/L, while mean effluent concentrations for the entire period were 420 ± 76, 165 ± 45 and 231 ± 64 μg/L for MG-Z, MG-R and MG-C, respectively. The inability of the MG-Z system to properly remove terbutylazine from wastewater was higher in the ET units than in the influent (with the highest values observed one year after the first sampling). The adsorption and degradation capacity of MG-Z support matrix underperformed to eliminate the influent concentration of terbutylazine. Higher terbutylazine effluent concentrations were measured during the summer period, when the temperature was high, which are attributed to condensation due to evapotranspiration (ET). According to a research that was conducted on the same pilot-scale units, higher ET rates were recorded during the warmer months [53]. More specifically, in the period from January to March the mean air temperature was 8.1 °C and the mean daily ET rates were 1.6, 1.0 and 1.3 (mm/d) for MG-Z, MG-R and MG-C, respectively; in the period from May to August, the mean air temperature was 25.1 °C, and the mean daily ET rates were 3.1, 12.3 and 12.8 (mm/d) for MG-Z, MG-R and MG-C, respectively. Similar observations were reported for triazines removal from horizontal subsurface flow CWs [54]. Furthermore, the accumulation of terbutylazine could also be ascribed to the delayed removal ability of the system, mainly due to the combined effect of the absence of vegetation, the finite adsorption capacity of the porous media and the short HRT (compared with the terbutylazine DT50 values). In addition, the presence of plants, compared to an unplanted CW, may promote microbial communities of different function and higher biofilm activity and metabolic richness [55].

Significantly lower removal efficiency was found in the unplanted CW MG-Z, which indicates that the presence of wetland plants (T. latifolia, P. australis) improves the pesticide treatment efficiency. No seasonal trend on fluctuation of terbutylazine concentrations in effluents of both MG-R and MG-C systems was observed. Similarly, Papavangelou et al. [21] reported the lack of seasonal variations in the influent and effluent concentrations of Nonylphenol and Bisphenol A. In most cases, the CW planted with P. australis (MG-R) showed the highest capability to remove terbutylazine from wastewater. The MG-R system reached up to 73.7% removal efficiency, while the highest efficiency of the MG-C system was 58.4% (Table 3; Fig. 3b). Terbutylazine is slowly degraded under aerobic aquatic conditions, in which chemical processes exert a more prominent role in the removal, compared to microbial degradation [56]. Morphological and physiological differences between the cattails and common reeds related to their ability to transfer oxygen to the rhizosphere could explain the differences observed between the two systems [40,57,58]. Moreover, differences in their exudation processes and related microbial community could affect degradation of terbutylazine within the rhizosphere [59–61]. The highest removal efficiency of both MG-R and MG-C systems was observed during the first sampling. Higher initial removal efficiency was also observed for boscalid in two HSF-CWs planted with P. australis [14], which indicates a higher initial adsorption capacity of the support matrix. However, the MG-R system retained its ability to remove high percentage of the applied terbutylazine for a longer period compared to the MG-C system. According to Matamoros et al. [62], terbutylazine showed high attenuation variability (i.e., 0–80%) in different CWs.

The Kruskal-Wallis test indicated statistically significant (p < .001) differences between the pilot-scale units in percent removal of terbutylazine. According to the Mann–Whitney U test, removal of terbutylazine in the MG-R unit was statistically significantly higher than that in MG-C (p < .001) and MG-Z (p < .001) units, and the removal of MG-C unit was statistically significantly higher than that in the MG-Z unit (p < .001).

Mean removal values in the MG-C at HRT of 6 and 8 days were 39.0% and 36.7%, respectively. Statistical analysis showed that there was no statistically significant difference between them (Mann–Whitney U test: p > .05). In the MG-R unit, the mean removal values at HRT of 6 and 8 days were 57.2% and 51.2%, respectively, and there was no statistically significant difference between them (p > .05). This indicates that a HRT of 6 days may be adequate for terbutylazine removal (Table 3).

Fig. 4 presents a chart relating terbutylazine removal efficiencies with effluent water temperatures. In MG-C and MG-R units, the slope of the trendlines is almost parallel to horizontal axis showing that the terbutylazine removal does not depend on temperature (Fig. 4). These graphs only show the trend and are not intended to show a relationship of high correlation between removal and temperature; for this reason, no equation and no correlation coefficient are presented. Statistical analysis also showed that there was no statistical significant correlation (p > .05) between removal and temperature for both MG-C and MG-R CWs.

Several studies highlighted the vegetation’s contribution to pesticide sorption and immobilization, breakdown or uptake [63]. Terbutylazine is a systemic herbicide absorbed principally through the roots with acropetally translocation and accumulation in the apical meristems and leaves. According to Papadopoulos et al. [31], the root system of Typha latifolia can absorb terbutylazine, while also acting as a pump by removing xenobiotics from soil. Mild phytotoxicity symptoms related to photosynthesis inhibition were observed during the last month of sampling in both cattails (T. latifolia) and common reed (P. australis). Terbutylazine content was determined in different plant compartments (roots, shoots and leaves) in December 2014 and September 2015 (Table 4). Results revealed higher concentrations in the second sampling of all plant materials, which followed the pattern.

### Table 3

| Removal % | MG-C | MG-R | MG-Z |
|-----------|------|------|------|
| EP 6 8   |      |      |      |
| Mean     | 37.6 | 39.0 | 36.7 |
| Max      | 58.4 | 58.4 | 55.1 |
| Min      | 8.6  | 21.7 | 8.6  |
| SD       | 11.9 | 12.6 | 11.5 |

EP: Experimental Period; 6 and 8 refer to the HRT.
roots > leaves > shoots; moreover, higher concentrations were detected in *P. australis* compared to *T. latifolia*, which also explains the higher removal rates in the MG-R. Concentrations detected were much higher than the EC_{50} values reported by others for *Lemna minor* and *Pseudokirchneriella subcapitata* [64]; however, the phytotoxicity of terbuthylazine to *P. australis* and *T. latifolia* was only observed after one year of full operation of the CWs. Although terbuthylazine can be rapidly metabolized in tolerant plants to various metabolites, the concentrations detected in plant material could affect the long-term efficiency and functionality of the CWs [65,66]. Terbuthylazine metabolites have been detected in material from *T. latifolia* by Papadopoulos et al. [31], and could contribute to the toxicological effects towards the plant.

Apart from phytoextraction, terbuthylazine adsorption on the sediment of a CW has been proposed as a major route of removal from wastewater, though reported concentrations of the herbicide and its metabolites were lower in sediments than within plants [3]. Although poor accumulation is expected in the gravel bed (substrate material) of the young CW systems, in older mature CW systems, as is the case of the present study (the CWs were about fourteen years old), greater adsorption is possible as organic matter concentration increases due to sedimentation of suspended solids and accumulation of leaf and stem residues in the substrate. Given that organic matter content in MG-R and MG-C systems was 1.6% and 1.8%, respectively, adsorption of terbuthylazine on the substrate cannot be excluded in our case. The influence of the organic matter content of mature CWs on the removal efficacy of boscalid has also been reported earlier [14]. Moreover, organic matter actually supports the microbial community and biodegradation conducted in the rhizosphere of *P. australis* and *T. latifolia*. According to Matamoros et al. [67], biodegradation and plant uptake are the most likely pathways for pesticide elimination in CWs with gravel bed. The composition of the support matrix material plays a major role through sorption phenomena and biodegradation, as also discussed below for the BPS [68].

### 3.2. Removal in biopurification system

Terbuthylazine removal in the biomixture exhibited an initial lag phase which lasted up to 4 d after pesticide application (Fig. 5a). Nonetheless, after that period, a steep elimination was observed, reaching 91.1% after 14 d and more than 98% after 28 d. Leaving aside the lag phase, the data was better described by a first order model with a half-life of 4.1 d, corresponding to 8.1 d since the beginning of the treatment. The promising results regarding removal and detoxification of terbuthylazine, provide a green light to employ these systems before the disposal of terbuthylazine-containing wastewaters in agricultural farms. Taking into consideration the terbuthylazine application indications, the volume of wastewater and the volume of a biopurification system, the concentrations of the pesticide employed were in the order of mg/kg. Terbuthylazine removal has been described in different biomixtures, nonetheless the half-lives reported in these systems have been consistently over 30 d. Kravariti et al. [69] achieved half-lives ranging from 99 d to 139 d in compost-based biomixtures, and Spliid et al. [38] reported a half-life of 60 d in a straw/sphagnum/soil full-scale biobed; moreover, removals of only 45% to 74% were obtained after one year in biomixtures containing alternative substrates such as coat pine and cork [70]. Faster elimination rates were described by Karanasios et al. [71,72], with half-lives as low as 30 d in compost-based matrices depending on the lignocellulosic substrate employed. In general, the elimination achieved in the present work is the fastest so far described for terbuthylazine in biomixtures. Moreover, the biomixture is also able to remove other triazines, including atrazine, ametryn and to a lesser extent terbutryn, as well as other herbicides such as linuron [35,37], thus increasing its potential usefulness. Interestingly, a similar matrix, bioaugmented with the ligninolytic fungus

---

**Table 4**

Terbuthylazine concentrations in plant material from the MG-R and MG-C systems.

| Plant material          | Concentration (mg/kg) | Date: 20/12/2014 | Date: 10/9/2015 |
|-------------------------|-----------------------|-------------------|------------------|
| *Phragmites australis* (leaves) | 2.25 (± 0.10)         | 3.81 (± 0.07)     |
| *Phragmites australis* (shoots) | 1.14 (± 0.09)         | 2.24 (± 0.07)     |
| *Phragmites australis* (roots) | 2.78 (± 0.08)         | 3.94 (± 0.09)     |
| *Typha latifolia* (leaves) | 1.18 (± 0.11)         | 2.10 (± 0.10)     |
| *Typha latifolia* (shoots) | 0.87 (± 0.04)         | 1.31 (± 0.06)     |
| *Typha latifolia* (roots) | 2.51 (± 0.01)         | 2.78 (± 0.05)     |

---

**Fig. 4.** Terbuthylazine removal rates (%) as function of water temperature in MG-C and MG-R pilot-scale CWs. Trendlines are also presented.

**Fig. 5.** Elimination of terbuthylazine and residual ecotoxicological analysis in a coconut fiber/compost/soil biomixture. (a) Profile of terbuthylazine concentrations in the biomixture. Values plotted are the means ± SD for triplicate samples. (b) Seed germination test with lettuce (*L. sativa*) as an indicator of phytotoxicity in elutriates from the biomixture during terbuthylazine removal. Relative seed germination (SG, ◦), relative root elongation (RE, ○) and germination index (GI, bars). Mean values significantly different from initial values (time 0 d) of each parameter are marked with an asterisk (*) (p < .05).
Trametes versicolor, could only remove 36.5% of the herbicide after 60 d [37]. Biological removal of triazines in BPS is mostly ascribed to bacteria hosting triazine-degrading genes, including atr (atrA to atrF), tri and trz [73]. Given the nature of biopurification systems, the pre-exposed soil used to prepare the biomixture provides most of the microbiota hosting these genes, and therefore, they have the ability to transform triazines such as terbutylazine.

According to our experimental conditions, the biomixture was loaded with high pesticide concentration (50 mg/kg). The performance of the biomixture was excellent, treating the highest pesticide concentrations found in agricultural wastewater. The lifetime of the biomixture is estimated at 6–8 years according to previous studies in temperate regions [74]; however, determinations in the tropics have suggested a significant loss in removal capacity after periods as short as 6 months [75]. Anyhow, the replacement of the lost volume due to degradation of organic matter can be easily done and it is a way to increase the lifetime of the system.

The terbutylazine containing biomixture proved to be non-toxic to D. magna, as demonstrated by an EC50 value of > 100 mg/L (i.e., < 1 TU) after addition of the pesticide in the matrix. The EC50 value reported in D. magna for this triazine is 21.2 mg/L [2], which is considered as moderately toxic. Maximum theoretical concentration in the elutriate is below this value (12.5 mg/L), but sufficient enough to have an effect; the lack of this effect strongly suggests that part of the terbutylazine was adsorbed to the biomixture, despite being moderately mobile (KOC = 231) [2]. In this respect, adsorption of pesticides is a desired characteristic in biomixtures, in order to reduce mobility towards the bottom of BPS, to avoid the formation of toxic gradients in the matrix [74]. As the removal took place, the EC50 value was kept constant for up to 28 d of treatment, indicating that potential transformation products were non-toxic or at least were not produced at large enough quantities to affect the microcrustaceans. Moreover, some of the metabolites described in soil, and likely to be produced in biomixtures as well, also exhibit moderate toxicity towards D. magna: desethyl-terbutylazine (EC50 > 42 mg/L), hydroxy-terbutylazine (EC50 > 2.8 mg/L), and 2-hydroxy-desethyl-terbutylazine (EC50 > 15 mg/L) [2].

Contrary to tests on D. magna, variations in the residual toxicity of the biomixture were observed during seed germination tests to determine phytotoxicity (Fig. 5b). Although slightly different values for SG, RE or GI parameters were achieved in most cases compared to the values at the moment of terbutylazine application (time 0), a decrease in the parameter values was noticed; in particular, RE was lower than the initial value after 31 d (Fig. 5b). The data suggest that even though removal of the herbicide is taking place in the biomixture, clear detoxification towards lettuce seeds was not achieved, maybe due to the formation of metabolites of unknown toxicity. Effects on non-target plants are not described for the above mentioned metabolites; however, moderate toxicity has been reported towards other photosynthetic organisms such as algae [2]. Ecotoxicological tests in more bioindicators are necessary to better describe the potential detoxification extent of the biomixture during the removal of terbutylazine.

The approaches employed in agricultural wastewater mitigation must be carefully designed to maximize removal, transformation and sorption of terbutylazine. In this respect, factors such as HRT, support matrix and plant species should be optimized in CWS; on the other hand, composition of biomixtures represents the most strategic way to optimize BPS. Based on the current findings, CWS would not be recommended to be used as sole management system of agricultural wastewater containing high concentrations of terbutylazine, as deleterious effects were observed on the plant compartment at long-term treatment. Similarly, despite the final removal in the BPS, the system failed to achieve a clear detoxification.

Recent studies suggest the use of complementary or coupled degradation technologies for the treatment of emerging pollutants (76,77). In particular, the use of hybrid CWS coupled to photodegradation processes has revealed increased removal efficiencies for several organic contaminants [78,79]. In the present case, the possibilities of using a combination of the two technologies should be considered. CWS could be used in series with a biomixture in order to increase the removal efficacy; given the fast removal in the biomixtures, and their use to treat wastewaters of high pesticide concentrations (around 10–50 mg/kg once disposed in the biomixture), this matrix could be employed as a pre-treatment, in order to obtain a flow of lower terbutylazine concentration before its entrance to the CWs, and therefore, applying a less toxic effluent to the plant compartment of the CW. The HRTs of 6 or 8 days could only be achieved by having controlled inflow working conditions, which are possible when, for example, spraying treating tank rinsing water. In the case when the aim of the CW is to directly treat agricultural runoff, a balancing tank and probably an appropriate hydraulic structure would be needed before the CW to control the influent in order to achieve the HRT of 6 or 8 days. Similarly, a combined configuration in which the biomixture is used as the support matrix in a CW (i.e., a free-water surface flow CW), could result in a system of increased removal efficiency. The support matrix is a vital component in HSF-CWs, because its characteristics influence the development of the biota, supply surface area for the development of microorganisms and stimulate their growth. However, the information available up to date on the microbial consortium developing in the various support matrices and their interaction with wetland rhizosphere is still rather limited [68]. The use of free-water surface flow (FWS) CWs could be even more promising and closer to the real conditions faced on point- and diffuse-source pollution by pesticides. Thus, further studies, including pesticides with different physiochemical properties, should be investigated in order to evaluate the performance of FWS CWs. The advantage of CWs and BPS relies on their cost-effectiveness and easy operation/maintenance for small farmers. The use of low cost materials (wastes) in their construction adds to their appeal as a way of residue re-valorization. Configurations should focus on assuring the detoxification of the wastewater, and for this, a wide range of bioindicators must be employed to obtain a better estimation of the eco-friendliness of the design process.

4. Conclusions

Two low-cost approaches were employed in the removal of terbutylazine from wastewaters. The herbicide was accumulated in the unplanted CW for both 6 and 8-day hydraulic residence times and increased concentrations were detected in the effluent wastewater resulting in negative removal. On the contrary, the CW planted with P. australis showed the highest terbutylazine removal capacity of up to 73.7%, while the CW planted with T. latifolia reached a maximum removal capacity of 58.4%. High concentrations of terbutylazine were detected in roots, leaves and shoots of both plants of the CWs, and mild phytotoxicity symptoms were observed after one year of full operation of the systems. On the other hand, the biomixture used in BPS efficiently removed terbutylazine, reaching 98% elimination in 28 d, faster than other reported biomixtures in the literature. However, the matrix was not capable to perform a clear detoxification, as demonstrated with seed germination tests. Potential configurations, combining CWs and BPS, can be used to increase the removal efficiency; nonetheless, careful optimization should focus to improving detoxification and supporting the activity of the depuration biota.

Acknowledgements

The authors acknowledge Vicerrectoría de Investigación, Universidad de Costa Rica (802-B4-503 and 802-B6-137), and the Costa Rican Ministry of Science, Technology and Telecommunications, MICTT (project FI-093-13). The secondments of researchers from Costa Rica to Greece for this project has received funding from the European Union’s Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 690618 KNOWPEC.
(Knowledge for pesticide control). The article reflects only the authors’ views and the Agency is not responsible for any use that may be made of the information it contains.

References

[1] D. Barceló, Occurrence, handling and chromatographic determination of pesticides in the aquatic environment, Analyst 116 (1991) 681–689.
[2] K.A. Lewis, J. Tzilivakis, D. Warner, A. Green, An international database for pesticide risk assessments and management, Hum. Ecol. Risk Assess. 22 (2016) 1050–1064.
[3] N.G. Papadopoulos, E. Gikas, G. Zalidis, A. Tsarbopoulos, Determination of herbicide terbutylazine and its major hydroxy and dealkylated metabolites in commercial wetland wastewater using solid phase extraction and high performance liquid chromatography–diode array detection, Int. J. Environ. Anal. Chem. 92 (2012) 1429–1442.
[4] A.P. Pinto, C. Serrano, T. Pires, E. Mestrinos, L. Dias, D. Martins, A.T. Caldeira, Degradation of terbutylazine, difenoconazol, and pendimethalin pesticides by selected fungus cultures, Sci. Total Environ. 435–436 (2012) 402–410.
[5] A. Claver, P. Ormad, L. Rodríguez, J.L. Oveilleiro, Study of the presence of pesticides in surface waters in the Ebro river basin (Spain), Chemosphere 66 (2006) 1437–1443.
[6] L.H. Du Preez, P.J. Van Rensburg, A.M. Jooste, J.A. Carr, J.P. Giesy, T.S. Gross, E.C. Kalogridi, C. Christophoridis, E. Bizani, G. Drimaropoulou, K. Fytianos, Part I: Analytical method, Environ. Sci. Pollut. R 21 (2014) 7139–7251.
[7] E.N. Papadakis, A. Tsaboula, A. Kotopoulou, K. Kintzikoglou, Z. Vryzas, G. Zalidis, A. Tsarbopoulos, Simultaneous determination of 21 pesticides in surface water using HSF and VF pilot-scale horizontal subsurface flow constructed wetlands, Environ. Pollut. 135 (2005) 131–141.
[8] A.L. Stefanakis, V.A. Tsihrintzis, Effects of loading, resting period, temperature, pesticide media, vegetation and season on performance of pilot-scale vertical flow constructed wetlands, Chem. Eng. J. 181 (2012) 416–430.
[9] C. Gregoire, D. Elnasser, H. Huguenot, J. Lange, T. Lebeau, A. Merli, R. Mose, E. Pasport, S. Payrasteau, T. Schutz, R. Schulz, G. Tapia-Pazda, J. Tournebize, M. Trevisan, A. Wanko, Mitigation of agricultural non-point-source pesticide pollution in artificial wetland ecosystems, Environ Chem. Lett. 7 (2008) 205–231.
[10] N. Papadopoulos, E. Gikas, G. Zalidis, A. Tsarbopoulos, Simultaneous determination of herbicide terbutylazine and its major hydroxy and dealkylated metabolites in T. lanfolia l. wetland plant using SPLC and HPLC-DAD, J. Liq. Chromatogr. & R. T. 30 (2009) 2957–2992.
[11] P. Schroder, H. Maier, R. Debus, Detoxification of herbicides in Phragmites australis, Z. Naturforsch. C 60c (2005) 317–324.
[12] K. Snigdowski, D. Springael, Establishment of multiple pesticide biodegradation capacities from pesticide-primed materials in on-farm biopurification system microcosms treating complex pesticide-contaminated wastewater, Pest Manage. Sci. 71 (2015) 986–995.
[13] C.E. Rodríguez-Rodríguez, C. Vasto-Gutiérrez, J.S. Chin-Pampillo, K. Ruiz-Hidalgo, On-farm biopurification systems: role of white rot fungi in depuration of pesticide-containing wastewaters, FEMS Microbiol. Lett. 345 (2013) 1–12.
[14] A. Huezo-Soto, H. Castro-Gutiérrez, M. Masís-Mora, J.S. Chin-Pampillo, C.E. Rodríguez-Rodríguez, Effects of oxytetracycline on the performance and activity of biomixtures: removal of herbicides and mineralization of chlorpyrifos, J. Hazard. Mater. 321 (2017) 1–8.
[15] A. Huezo-Soto, M. Masís-Mora, V. Lizano-Fallas, J.S. Chin-Pampillo, E. Carazo-Rojas, C.E. Rodríguez-Rodríguez, Simultaneous removal of structurally different pesticides in a biomixture: detoxification and effect of oxytetracycline, Chemosphere 169 (2017) 558–567.
[16] V. Lizano-Fallas, M. Masís-Mora, D. Espinoza-Villalobos, M. Lizano-Brenes, C.E. Rodríguez, Removal of pesticides and ecotoxicological changes during the simultaneous treatment of triazines and chlorpyrifos in biomixtures, Chemosphere 182 (2017) 106–113.
[17] N.H. Spilioti, A. Helweg, K. Heinrichson, Leaching and degradation of 21 pesticides in a full-scale model biobed, Chemosphere 65 (2006) 2223–2232.
[18] K. Ruiz-Hidalgo, J.S. Chin-Pampillo, M. Masís-Mora, E. Carazo-Rojas, C.E. Rodríguez-Rodríguez, Degradation of carbamoyl by Trametes versicolor in rice husk as a potential lignocellulosic substrate for biomixtures: from mineralization to toxicity reduction, Process Biochem. 49 (2014) 2266–2271.
[19] C.S. Akratos, V.A. Tsihrintzis, Effect of temperature, HRT, vegetation and porous media on removal efficiency of pilot-scale horizontal subsurface flow constructed wetlands, Ecol. Eng. 29 (2007) 173–191.
[20] P. Verlicchi, E. Zambello, How efficient are constructed wetlands in removing pharmaceuticals from untreated and treated urban wastewaters? A review, Sci. Total Environ. 470–471 (2014) 1281–1296.
[21] J.S. Chin-Pampillo, K. Ruiz-Hidalgo, M. Masís-Mora, E. Carazo-Rojas, C.E. Rodríguez-Rodríguez, Adaptation of biomixtures for carbamoyl degradation in on-farm biopurification systems in tropical regions, Environ. Sci. Pollut. Res. 22 (2015) 9869–9884.
[22] J.S. Chin-Pampillo, K. Ruiz-Hidalgo, M. Masís-Mora, E. Carazo-Rojas, C.E. Rodríguez-Rodríguez, Design of an optimized biomixture for the degradation of carbamoyl based on pesticide removal and toxicity reduction of the matrix, Environ. Sci. Pollut. R. 24 (2017) 19184–19193.
[23] EC, Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed. SANTE/11945/2015, Directorate-General for Health and Food Safety, (2015).
[24] J. Liu, L. Tong, D. Li, W. Meng, W. Sun, Y. Zhao, Z. Yu, Comparison of two extraction methods for the determination of 135 pesticides in Coralidis Rhizoma, Chuanxiong Rhizoma and Angelicae Sinensis Radix by liquid chromatography–triple quadrupole–mass spectrometry: Application to the roots and rhizomes of Chinese herbal medicines, J. Chromatogr. A 1807–1818 (2018) 233–246.
[25] EPA, EPA-823-B-01-002 Methods for collection, storage and manipulation of sediments for chemical and toxicological analyses: Technical Manual. Office of Water (4305), Washington, DC. (2001).
[26] USEPA, EPA-823-R-02-001 Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. Office of Water (4303T), Washington, DC. (2002).
[27] USEPA, USEPA-820-R-00-003 Seed Germination/Root Elongation Toxicity Test. OPPTS Ecological Effect Guidelines, 850 Series, (1996).
[28] T. Lv, Y. Zhang, L. Zhang, P.N. Carvalho, C.A. Arias, H. Brix, Removal of the pesticides imazalil and tebuconazole in saturated constructed wetland mesocosms, Water Res. 91 (2016) 628–638.
[29] R.C. Robinson, R.J. Dunham, The uptake of soil-applied chlorotetracyclines by seedlings and its prediction, Water Res. 22 (1982) 223–236.
[30] J. Fenoll, N. Vela, G. Navarro, P. Perez-Lucas, S. Navarro, Assessment of agro-industrial and composted organic wastes for reducing the potential leaching of triazine herbicide residues through the soil, Sci. Total Environ. 493 (2014) 124–132.
[31] F. Sichizadi, G.A. Sacchi, M. Trevisan, A.M. Del Re, Root uptake and xylem translocation of pesticides from different chemical classes, Pest. Sci. 50 (1997) 111–119.

G.D. Gikas et al.
Chemical Engineering Journal 335 (2018) 647–656
[53] V.A. Papaevangelou, G.D. Gikas, V.A. Trihstinris, Evaluation of evapotranspiration in small on-site HSF constructed wetlands, J. Environ. Sci. Health A Toxicol. Hazard. Subst. Environ. Eng. 47 (2012) 766–785.

[54] J. Wu, Z. Li, L. Wu, F. Zhong, N. Cai, Y. Dai, S. Cheng, Triazophos (TAP) removal in horizontal subsurface flow constructed wetlands (HSCWs) and its accumulation in plants and substrates, Sci. Rep. 7 (2017) 5468.

[55] T. Lv, P.N. Carvalho, L. Zhang, Y. Zhang, M. Button, C.A. Arias, K.P. Weber, H. Irix, Functionality of microbial communities in constructed wetlands used for pesticide remediation: influence of system design and sampling strategy, Water Res. 110 (2017) 241–251.

[56] L. Delgado-Moreno, A. Pena, Compost and vermicompost of olive cake to bioremediation of soils contaminated by atrazine, Int. J. Phytoremediation 4 (2002) 1–15.

[57] M.P. Castillo, L. Torstensson, J. Stenstrom, Biobeds for environmental protection of industrial areas with emphasis on the importance of the support matrix, J. Hazard. Mater. 252 (2013) 272–292.

[58] L. Wackett, M. Sadowsky, B. Martinez, N. Shapir, Biodegradation of atrazine and related s-triazine compounds: from enzymes to field studies, Appl. Microbiol. Biotechnol. 58 (2002) 39–48.

[59] B. Du, A.E. Price, W.C. Scott, L.A. Kristofco, A.J. Ramirez, C.K. Chambles, J.C. Yelderman, B.W. Brooks, Comparison of contaminants of emerging concern removal, discharge, and water quality hazards among centralized and on-site wastewater treatment systems,民眾收購使用於禾本間的石隔, Chemosphere 80 (2010) 418–425.

[60] V. Castro-Gutiérrez, M. Masís-Mora, M.C. Diez, G.R. Tortella, C.E. Rodríguez, Aging of biomixtures: evaluation for use in biobed systems, Chemosphere 168 (2017) 418–425.

[61] J.L. Bouldin, J.L. Farris, M.T. Moore, S. Smith, W.W. Stepens, C.M. Cooper, Evaluated fate and effects of atrazine and lambda-cyhalothrin in vegetated and unvegeted microcosms, J. Environ. Toxicol. 20 (2005) 487–498.

[62] N. Gedergreen, J.C. Steehig, The toxicity of herbicides to non-target aquatic plants and algae: assessment of predictive factors and hazard, Pest. Manage. Sci. 61 (2005) 1152–1160.

[63] E. Gikas, N.G. Papadopoulos, F.N. Bazioti, G. Zaldívar, A. Tsaropoulos, Use of liquid chromatography/electrospray ionization tandem mass spectrometry to study the degradation pathways of terbuthylazine (TER) by T. infelix in constructed wetlands: identification of a new TER metabolite, Rapid Commun. Mass Sp. 26 (2012) 181–188.

[64] K. Krzew, R. Tommasini, E. Martinoia, Old enzymes for a new job (Herbicide detoxification in plants), Plant Phys. 111 (1996) 349–353.

[65] V. Matamoros, J. Puigagut, J. García, J.M. Bayona, Behavior of selected priority organic pollutants in horizontal subsurface flow constructed wetlands: a preliminary screening, Chemosphere 69 (2007) 1374–1380.

[66] A.V. Dordor, A.J.P. Carvalho, Organic xenobiotics removal in constructed wetlands, with emphasis on the importance of the support matrix, J. Hazard. Mater. 252–253 (2013) 272–292.

[67] J. Vymazal, The use of hybrid constructed wetlands for wastewater treatment with special attention to nitrogen removal: a review of a recent development, Water Res. 47 (2013) 4795–4811.