Czech University
of Life Sciences Prague

Effects of arbuscular mycorrhizal fungi on the removal of emerging organic pollutants in constructed wetlands

Ph.D. thesis

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Thesis

This thesis is submitted in fulfillment of the requirements for the Ph.D. degree at the Czech University of Life Sciences Prague, Faculty of Environmental Sciences.

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Abstract

The continuous discharge of emerging organic pollutants (EOPs) into the environment and their potential biotoxicity to aquatic organisms has attracted increasing concerns. Constructed wetlands (CWs) are considered to be a sustainable technology for EOPs removal. Still, long-term exposure to EOPs and their metabolites may also negatively affect plant growth and system stability. Arbuscular mycorrhizal fungi (AMF), as the most common microorganism in the natural environment, have positive effects on the performance of the host plant through increasing nutrient uptake and tolerance to environmental stresses, including EOPs. Consequently, AMF has been regarded as a critical phytoremediation approach to re-establish the degraded ecosystems. However, little is known about the application of AMF in CWs to purify wastewater containing EOPs. Furthermore, factors that affect the establishment of AM symbiosis (e.g., substrate types) in wetland systems remain unclear. Therefore, the main objectives of this thesis were to: 1) assess the impacts of substrate type (sand, perlite, vermiculite, or biochar) on the symbiotic relationships between AMF (*Rhizophagus irregularis*) and plant roots (*Glyceria maxima*) in CWs; 2) explore the effects of AM symbiosis on plant growth and antioxidant response in CWs under the stress of EOPs; 3) investigate the influences of AMF on the transformation and translocation of EOPs in CWs; 4) evaluate the role of AMF in pollutant removal in CWs with the different substrate. Results showed that the symbiosis between AMF and *G. maxima* could be found in all substrate CW systems. Adsorptive substrates (perlite, vermiculite, and biochar) were more conducive to forming AM symbiosis than sand. AMF significantly enhanced the growth of *G. maxima*, and promoted host plant resistance to EOPs' stresses by increasing antioxidant enzymes and soluble protein contents in plant tissues and decreasing oxidative damage. Meanwhile, AM symbiosis enhanced the translocation of EOPs from roots to shoots in all substrate systems, resulting in a higher amount of EOPs in the shoots of inoculated plants than that of non-inoculated plants. Compared to the non-AMF inoculated controls, The accumulation of EOPs in the roots of inoculated plants was increased by 21-193% and 67-196% in sand and vermiculite systems but decreased 13-55% and 51-100% in perlite and biochar systems, respectively. AMF had positive effects on pollutant removal (EOPs and conventional pollutants such as TOC, PO_4^{3-}-P, NH_4^+-N, TN) in sand systems but insignificant effects in perlite, vermiculite, and biochar systems. In addition, AM symbiosis effectively reduced the concentration of EOPs metabolites (2-hydroxy ibuprofen, carboxy ibuprofen, and 4'-hydroxy diclofenac) in the effluent of all four substrate systems. Therefore, these results indicated that AMF has the potential to enhance the performance of CWs for wastewater purification, and this ability could be affected by substrate types. These may provide a good starting point for applying AMF in the phytoremediation of EOPs in CW systems.

Abstrakt

Kontinuální vypouštění nově se objevujících organických polutantů (EOP) do životního prostředí a jejich potenciální biotoxicita pro vodní organismy vzbuzuje stále větší obavy. Vybudované umělé mokřady (UM) jsou považovány za udržitelnou technologii pro odstraňování EOPs. Dlouhodobá expozice EOP a jejich metabolitům však může také negativně ovlivnit růst rostlin a stabilitu systému. Arbuskulární mykorhizní houby (AMF), jako nejběžnější mikroorganismus v přirozeném prostředí, mají pozitivní vliv na výkonnost hostitelské rostliny prostřednictvím zvýšeného příjmu živin a tolerance vůči environmentálním stresům, včetně EOP. V důsledku toho byl AMF považován za kritický fytoremediační přístup k obnovení degradovaných ekosystémů. O použití AMF v UM k čištění odpadních vod obsahujících EOP je však známo jen málo. Kromě toho zůstávají faktory, které ovlivňují ustavení AM symbiózy (např. typy substrátů) v mokřadních systémech, nejasné. Hlavními cíli této práce proto bylo: 1) posoudit dopady typu substrátu (písek, perlit, vermikulit nebo biouhel) na symbiotické vztahy mezi AMF (*Rhizophagus regularis*) a kořeny rostlin (*Glyceria maxima*) v UM; 2) prozkoumat účinky AM symbiózy na růst rostlin a antioxidační reakci u UM pod stresem EOP; 3) zkoumat vlivy AMF na transformaci a translokaci EOP v UM; 4) vyhodnotit úlohu AMF při odstraňování znečišťujících látek v UM s různým substrátem. Výsledky ukázaly, že symbiózu mezi AMF a *G. maxima* lze nalézt ve všech substrátech použitých v UM. Adsorpční substráty (perlit, vermikulit a biouhel) více napomáhaly formování AM symbiózy než písek. AMF významně zvýšily růst *G. maxima* a podpořily odolnost hostitelské rostliny vůči stresu EOPs zvýšením antioxidačních enzymů a obsahu rozpustných proteinů v rostlinných tkáních a snížením oxidačního poškození. AM symbióza navíc zlepšila translokaci EOPs z kořenů do výhonků ve všech použitých substrátech, což vedlo k vyššímu množství EOPs ve výhoncích inokulovaných rostlin než u neočkovaných rostlin. Ve srovnání s kontrolami neočkovanými AMF se akumulace EOP v kořenech inokulovaných rostlin zvýšila o 21–193% a 67–196 % v UM s pískem a s vermikulitem, ale snížila se o 13–55% a 51–100% v perlitu a v biouhlu (biocharu), resp. AMF měla pozitivní účinky na odstraňování polutantů (EOPs a konvenční polutanty jako TOC, PO_4^{3-}-P, NH_4^+-N, TN) v pískových systémech, ale nevýznamné účinky v perlitu, vermikulitu a biouhlu. AM symbióza navíc účinně snižovala koncentraci metabolitů EOPs (2-hydroxyibuprofenu, karboxyibuprofenu a 4'-hydroxy diklofenaku) ve výtocích ze všech čtyř UM s různými substráty. Tyto výsledky tedy naznačují, že AMF má potenciál zvýšit výkon UM pro čištění odpadních vod a tato schopnost může být ovlivněna typy substrátů. Ty mohou poskytnout dobrý výchozí bod pro aplikaci AMF ve fytoremediaci EOP v umělých mokřadech.

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Chapter I

Introduction

1. Introduction

More than 1000 emerging organic pollutants (EOPs), which are currently not added into the traditional monitoring project by many countries, have been detected in the environment according to the investigation of Norman Network (NORMAN Association, 2016). Many EOPs exhibit potential toxic effects on aquatic organisms due to their characteristics of bioaccumulation and difficulty for biodegradation (Hao et al., 2007), which could eventually pose a risk to human health and the ecosystem (Chen et al., 2017). Especially for some daily pharmaceuticals with a huge consumption, such as ibuprofen (IBU) and diclofenac (DCF). They can be released continuously to the aquatic ecosystem, for example, non-negligible levels of IBU and DCF have been found in the range of 1.7-373 μg/L and 0.7-48 μg/L in the influent of the wastewater treatment plant, respectively (Madikizela and Chimuka, 2017; Petrie et al., 2015). There are highly environmental concerns on IBU and DCF for aquatic life, including producing sex-specific responses (Flippin et al., 2007), genotoxic effects (Ragugnetti et al., 2011), influencing hatching, yolk sac, and tail deformation (Brandhof and Montforts, 2010). Moreover, conventional wastewater treatment plants have been proved to be one of the significant sources to discharge EOPs into surface water (Farré et al., 2008). Therefore, developing more effective wastewater treatment technology is an urgent need to prevent the release of EOPs into ecosystems.

Constructed wetlands (CWs), also known as treatment wetlands, are engineered systems designed to simulate various physical, chemical, and biological processes to remove pollutants (Vymazal, 2011). They have been wildly adopted as a cost-effective and sustainable technology for EOPs removal because of the advantages in low energy requirements, easy operation, and maintenance (Zhang et al., 2018). However, wetland plants and microbes are frequently exposed to the stress of EOPs, which may also affect plant growth and microbial activities as well as their subsequent performance to remove pollutants (Ilyas and van Hullebusch, 2020; Ji et al., 2022). A recent study reported by Díaz-Cubilla et al. (2022) revealed that the presence of EOPs could produce microbial stress and irreversible cell damages of microbes, influencing the anaerobic digestion process of anaerobic bioreactor and decreasing the removal efficiency of organic matters. Meanwhile, EOPs might have adverse effects on plant growth, such as reducing photosynthetic pigments, inhibiting root development, and reducing the number and size of mature leaves (Bartrons and Peñuelas, 2017). Therefore, there is an

urgent need to enhance plant tolerance to EOPs' stress, thus improving the performance and sustainability of CWs.

Arbuscular mycorrhizal fungi (AMF), as the most common microorganism in the natural environment, have been demonstrated to form a mutualistic symbiosis with roots of over 80% of all terrestrial plants. The plant-mycorrhizal association can develop the exraradical mycelium beyond the root-hair zone and establish tree-shaped subcellular structures within root cells, thus, improving nutrient acquisition and plant tolerance to environmental stresses, including drought, cold, salinity, and organic contaminants (Ajit Varma, 2017; Xu et al., 2017). Meanwhile, arbuscular mycorrhizal (AM) symbiosis also plays a significant role in boosting the development of rhizosphere microorganisms by stimulating the production of root exudates, phytoalexins, and phenolic compounds (Latef et al., 2016; Toljander et al., 2007). For these reasons, AMF has been regarded as a critical phytoremediation approach to re-establish the degraded ecosystems, such as contaminated soils, abandoned agricultural fields, and grassland (Ajit Varma, 2017). Previous studies indicated that AMF could influence the biodegradation and phytoremediation of EOPs, such as improving the removal of polycyclic aromatic hydrocarbons and petroleum compounds in soil (Małachowska-Jutsz and Kalka, 2010; Yu et al., 2011). Besides, Fester (2013) reported that AMF (*Funneliformis mosseae* and *Rhizophagus irregularis*) could rapidly and extensively establish symbiosis in the roots of *Phragmites australis* under the stress of benzene, methyl *tert*-butyl ether, and ammonia in a pilot-scale CW. Nowadays, numerous pieces of evidence have demonstrated that the symbiotic relationships between AMF and plant roots can be found from various wetland habitats, including fens, swamps, marshes, shorelines, bays, floating wetland mats, and natural wetlands (Fusconi and Mucciarelli, 2018; Huang et al., 2021; Ramírez-Viga et al., 2020; Wang et al., 2018a). This indicates that AMF may have an essential function in wetlands, which might open new perspectives on the application of symbiotic AMF in the phytoremediation technology of wastewater contaminated with EOPs. However, there is no information so far about the effects of AMF on the removal of EOPs in CWs.

Therefore, the objectives of this thesis were to: 1) assess the impacts of substrate type on the symbiotic relationships between AMF and plant roots in CWs; 2) explore the effects of AM symbiosis on plant growth and antioxidant response in CWs under the stress of EOPs; 3) investigate the influences of AMF on the transformation and translocation of EOPs in CWs; 4) evaluate the role of AMF in pollutant removal in CWs with the different substrate.

Chapter II

State of the art

Contents

2.1 Emerging organic pollutants

In recent decades, many newly identified compounds in the aquatic environment have become a global issue of increasing environmental concern (Rout et al., 2021). These contaminants are anthropogenic or natural and primarily organic, occurring in trace concentrations ranging from ng/L to μg/L (Rodriguez-Narvaez et al., 2017). These organic compounds are often collectively referred to as emerging organic pollutants (EOPs) (Farré et al., 2008). The United States Geological Survey defines EOPs as any anthropogenic or naturally occurring chemical that is not currently covered by existing water-quality regulations but has the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects. As shown in **Table 2.1**, EOPs arise from diverse groups of chemicals and various compounds, including pharmaceuticals and personal care productions (Dalahmeh et al., 2020; Madikizela and Chimuka, 2017), industrial additives and agents (Vymazal et al., 2017; Wang et al., 2021), perfluorinated alkylated substances, artificial sweeteners and biocides (Rathi et al., 2021; Robles-Molina et al., 2014), as well as other organic contaminants (Rout et al., 2021).

The existences of EOPs in environmental media are not a new phenomenon. The release of EOPs in the environment has occurred for a long time but might not have been reported until new analytical techniques were developed. The first documented awareness of EOPs, for example, should probably be attributed to the book published in 1962 by Rachel Carson, named “Silent Spring”, which focused on the relationship between widespread usage of dichlorodiphenyltrichloroethane (DDT) and ecological hazards (Sauvé and Desrosiers, 2014). In 1975, the first detection of pharmaceuticals was reported in surface water using gas chromatography with mass spectrometry (Rout et al., 2021). In recent years, with rapid advances in analytical techniques and detection methods, there has been a wealth of studies on the occurrence and fate of EOPs in the environment (Matamoros et al., 2012; Rodriguez-Mozaz et al., 2015; Verlicchi et al., 2012; Walters et al., 2010). With the industrial developments and technological advancements, the continuous release of various types of EOPs into the environment is a growing concern because they can be doubtlessly dangerous to the ecosystem and human beings (Farré et al., 2008; Gavrilescu et al., 2015; Wang et al., 2021).

Table 2.1. General classification of EOPs.

Classification	Representative EOPs
Pharmaceuticals	Ibuprofen, Diclofenac, Gemfibrozil, Diazepam, Aspirin, Acetaminophen, Sulfamethazine, Sulfamethoxazole, Naproxen, Carbamazepine, Ketoprofen, Chloramphenicol, Acetylsalicylic Acid, Diazepam, Furosemide, Bezafibrate, Hydrochlorothiazide, Xenoestrogen, Bisphenol A (BPA), Dioctyl phthalate, Amphetamine, Cocaine, Tetrahydrocannabinol, Diethylstilbestrol, Estradiol, Estriol, and Estrone
Personal care products	Benzophenone, N, N-diethyltoluamide, Methyl-benzylidene, Nitro, Polycyclic and Macrocyclic musks, Triclosan, Triclocarban, and Caffein
Industrial additives and agents	Chelating agents, Aromatic sulfonates, Dialkyl ethers, Methyl-t-butyl ether, Dimethyl adipate, C10-C13 chloroalkanes, Hexabromocyclododecane, Polybrominated diphenyl ethers, Tris (1-chloro-2-propyl) phosphate, Tetrabromo bisphenol A, Tris (2-chloroethyl) phosphate, Alkylphenol ethoxylates, Alkylphenols (nonylphenol and octylphenol), and Alkylphenol carboxylates
Perfluorinated alkylated substances	Perfluorooctanesulfonate and Perfluorooctanoic acid
Biocides	Epoxiconazole, Butachlor, and Metaldehyde
X-ray contrast agents	Iohexol, Iopromide, Iodixamol, Ioxaglate, Iothalamate, and Iopamidol
Artificial Sweeteners	Aspartame, Saccharin, and Sucralose
Other chemicals	Nanomaterials, 1,4-dioxane, and Swimming-pool-disinfection by-products

The information in this table is summarized from the following studies (Dalahmeh et al., 2020; Madikizela and Chimuka, 2017; Rathi et al., 2021; Robles-Molina et al., 2014; Rout et al., 2021; Vymazal et al., 2017; Wang et al., 2021).

2.1.1 Sources and occurrences of EOPs in aquatic environment

The occurrence of EOPs has been demonstrated in the environment worldwide (Pal et al., 2010). In addition to raw and treated wastewater (Mladenov et al., 2022), several studies have reported the presence of EOPs in surface water (Gorito et al., 2018), groundwater (Lapworth et al., 2012), and even drinking water (Teodosiu et al., 2018). According to the latest investigation data of Norman Network (NORMAN Association, 2016), more than 1000 EOPs that are currently not added into the traditional monitoring project by many countries have been detected in the aquatic environment.

EOPs can enter the environment through diversified sources (**Fig. 2.1**), which could be divided into two categories: point sources and diffuse sources of pollution (Lapworth et al., 2012). Point source pollution of EOPs usually originates from discrete locations, including effluents of industrials (e.g., hospitals, manufacturing plants, food processing plants) and municipal wastewater treatments, overflows of combined sewage-storm water, waste disposal sites (e.g., farm waste lagoons, landfill, industrial impoundment), resource extraction (e.g., oilfield and mining) and buried septic tanks. Diffuse pollution of EOPs, in contrast, is mainly of unspecified diffuse sources and usually occurs over broad geographical secles. The typical examples of diffuse source pollution include diffuse aerial deposition, agricultural runoff from bio-solids and manure sources, leakage from reticulated urban sewer systems, and stormwater and urban runoff (Lapworth et al., 2012).

EOPs in surface water

Out of the extensive classifications of EOPs detected in surface water (**Table 2.1**), Das (2017) suggested that about 30% are agricultural and industrial chemicals, and 70% are pharmaceuticals and personal care products (PPCPs). Among the various sources, the discharge of the effluent of wastewater water treatments (WWTPs) is one of the major sources of EOPs into the environment. For example, pharmaceuticals and their metabolites can be excreted via urines and feces and subsequently discharged into the environment through the effluents of WWTPs. Kümmerer (2009) suggested that even

a seemingly insignificant source such as private households can increase the level of EOPs in the environment by disposing of expired and unwanted medicines via household waste, sink, or toilet. Meanwhile, personal care products, such as shampoos, soap, shower gel, toothpaste, sunscreen lotions, etc., can be discharged to the environment through the daily activities of human beings (Yang et al., 2017). Kapelewska et al. (2018) investigated the occurrence of EOPs (nineteen compounds from different classifications) in three WWTPs, suggesting that the most frequently detected EOPs in the influent of WWTPs were PPCPs (52%), followed by industrial compounds (13%) and insect repellents (below 2%). Commonly, the degradation of EOPs in WWTPs is often incomplete. As shown in **Table 2.2**, most of the PPCPs were detected at levels below 5 μg/L in treated wastewater. However, some exceptions such as ibuprofen, diclofenac, naproxen, and caffeine were reported at concentrations of 603 μg/L, 270 μg/L, 70 μg/L, and 50 μg/L, respectively (Rout et al., 2021). So far, the effluent of WWTPs has become one of the primary sources of EOPs to discharge into the aquatic environment (Farré et al., 2008). The indirect or direct discharge from wastewater sources, such as manufacturing units of pharmaceuticals, personal care products, biocides, industrial compounds, and other chemicals, is considered another essential point source of EOPs in the aquatic environment (Jurado et al., 2012; Lapworth et al., 2012), especially in regions where discharge guidelines do not exist, and wastewater facilities are poorly regulated, rudimentary or non-existent. For example, the effluents of hospitals form a vital source for a range of specific EOPs, including antibiotic resistance bacteria/genes, x-ray contrast media, drug conjugates, pharmacy metabolites, etc. (Lapworth et al., 2012; Rout et al., 2021). Moreover, as intensive livestock rearing and highly mechanized farming become more and more common, the runoff from livestock farming and agricultural activities are gradually becoming a significant source of EOPs to the environment, particularly in the form of steroid hormones and pesticides used for the enhancement of productivity (Barbosa et al., 2016). Kolodziej and Sedlak (2007) found that the source of steroid hormones (e.g., estrogens, progestins, and androgens) in the surface water was directly co-related to livestock grazing. In dairy waste lagoons. Similarly, Kolodziej et al. (2004) reported that some hormones, including endogenous oestrogens, oestrone, androgens testosterone, androstenedione, have been detected at concentrations as high as 650 ng/L. Other sources of EOPs to the surface water include leakage from WWTP facilities, the discharge of aquaculture wastewater, irrigation using reclaimed water, landfill leaching, etc. (Rout et al., 2021; Yang et al., 2017). In a recent study, Nika et al. (2020) confirmed that samples from raw and treated landfill leachate yielded 58 complex EOPs involving pharmaceuticals, industrial chemicals, and plant protection products.

Table 2.2. The detected concentrations of PPCPs in the aquatic environment.

Compounds	Range in concentrations (ng/L)					
	Europe		**North and South America**		**Asia and Australia**	
	WWTP/STP	Surface water	WWTP/STP	Surface water	WWTP/STP	Surface water
Antibiotics						
Trimethoprim	In: - Out:0.5-7900	2-212	In: - Out: 58-321	4-150	In: - Out: 99-1264	0-78.2
Ciprofloxacin	In: - Out:110-1100	-	In: - Out: 42-720	23-1300	In: - Out: 40-3353	-
Sulfamethoxazole	In: - Out:7-211	91-794	In: - Out: 2-2000	2000	In: - Out: 0.5-4	3.8-1400
Analgesics						
Naproxen	In: 648-7264 Out: 1-500	0-979	In: 2.6×10^{3}-4.6×10^{6} Out: 128-7×10^{4}	11-3990	In: - Out: 450-1840	0.3-146
Ibuprofen	In: 540-2.0×10^{4} Out: 220-3600	0-34.0	In: 2.0×10^{3}-2.3×10^{6} Out: 65-1758	28-1025	In: 0-155 Out: 134-7100	14-83.4
Ketoprofen	In: 230-1288 Out: 12-298	17-737	In: 80 Out: 50	0.4-79.6	In: - Out: 225-954	9-14
Diclofenac	In: 90-1600 Out: 250-1310	11-1153	In: 1.7×10^{2}-2.5×10^{6} Out: 1.3×10^{3}-2.7×10^{5}	1.1- 1.9×10^{5}	In: 31.7 Out: 460-3300	21-41

Compounds	Range in concentrations (ng/L)					
	Europe		**North and South America**		**Asia and Australia**	
	WWTP/STP	Surface water	WWTP/STP	Surface water	WWTP/STP	Surface water
Paracetamol	In: 2.0×10^3-7.0×10^5 Out: 27-55	250-1289	In: 1.3×10^5 Out: -	4.2×10^4	In: 1350 Out: 17	18.7
Mefenamic acid	In: - Out: -	-	In: - Out: 4.45-396	0.1-65.1	In: - Out: 1-554	0.3-169
Acetaminophen	In: 4.9×10^4-1.1×10^5 Out: 1234-15947	24.7-954	In: 1.2×10^4-9.2×10^4 Out: 1.8-19	4.1-3422	In: - Out: 59-220	12-777
Antiepileptics						
Carbamazepine	In: 26-589 Out: 111-187	2.7-113.7	In: 290 Out: 152-226	25-34.7	In: 12-45 Out: 130-290	9-157
Beta-blockers						
Propranolol	In: 11-53 Out: 8-1350	2-54	In: - Out: 50-215	10.4	In: - Out: 30-44	20
Metoprolol	In: 186-5242 Out: 351-3097	0.8-115	In: - Out: 574-6990	26.3	In: 122 Out: 126	3.08
Atenolol	In: 160-9929 Out: 879	6.2-470	In: - Out: 45-3230	27-51	In: 1300 Out: 1720	0.35-314
Blood lipid regulators						

Compounds	Range in concentrations (ng/L)					
	Europe		**North and South America**		**Asia and Australia**	
	WWTP/STP	Surface water	WWTP/STP	Surface water	WWTP/STP	Surface water
Clofibric acid	In: - Out: 0-33	3.2-26.7	In: - Out: 154	22-248	In: - Out: 27-120	1-14
Gemfibrozil	In: - Out: 9-300	5.4-16	In: - Out: 3.9-17	1.8-9.1	In: - Out: 2-28571	-
Bezafibrate	In: 3-1369 Out: 3-302	4.05-328	In: 3150 Out: 748	1513	In: - Out: 233-340	16-363
Hormones						
Estriol	In: 5-102 Out: 5-85	0.2-34	In: 15-274 Out: 8.9-2.5×10^5	1.7-2510	In: 6.7-94 Out: 2.8-85	7.6
Estrone	In: 9-78 Out: 1-54	0-76	In: 21 Out: 14	3.6-34	In: 24.78-80 Out: 12.4-196.7	0.4-33
Progesterone	In: - Out: -	14-27	In: - Out: 9	-	In: - Out: 8.0-16.9	-

Note: WWTP: wastewater treatment plant. STP: sewage treatment plant. Dashed line-not reported. Data were summarized from previous studies (Jurado et al., 2012; Köck-Schulmeyer et al., 2013; Mladenov et al., 2022; Rúa-Gómez and Püttmann, 2012; Santos et al., 2007).

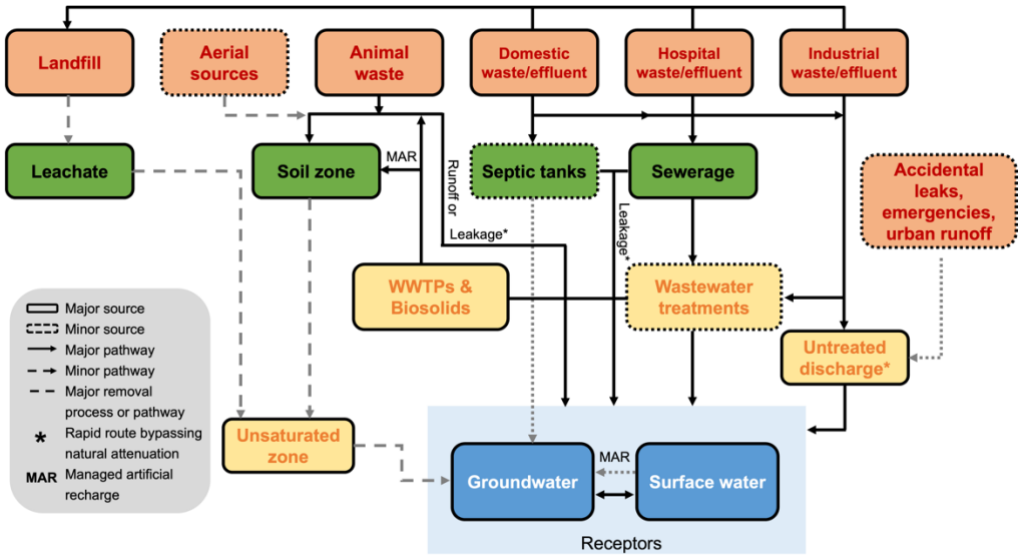

Fig. 2.1. The potential sources and pathways of emerging organic pollutants in the environment (Lapworth et al., 2012).

To date, more than 200 pharmaceutically active compounds have been reported globally in surface water (Dos Santos et al., 2021; Hughes et al., 2013). The detected concentrations of PPCPs in the effluent and influent of wastewater/sewage treatments and surface water are summarized in **Table 2.2**. For example, sulfamethoxazole, a sulfonamide, is one of the ubiquitously used antibiotics, and it was detected frequently in surface water in the range of 1.7-2000 ng/L (Pal et al., 2010). Ciprofloxacin, an antibiotic compound, was detected in the freshwater ecosystems with a maximum concentration of 6.5 mg/L (Hughes et al., 2013). Likewise, naproxen, ibuprofen, diclofenac, and paracetamol, as the commonly used analgesics, antiarthritic and antirheumatic non-steroidal anti-inflammatory drugs, have been regarded as the most frequently detected PPCPs in the aquatic environment, with a high concentration of 4.0 µg/L, 1.0 µg/L, 200 µg/L, and 40 µg/L, respectively (Palma et al., 2020; Veras et al., 2019). In a previous review conducted by Jones et al. (2001), it was found that the detected concentrations of PPCPs, including antibiotics, hormones, antidepressants, lipid regulators, analgesic compounds, and chemotherapy compounds, were ranged from 0.04 to 6.3 µg/L in the aquatic environment. Moreover, some high usage compounds like sunscreen agents and preservatives were often detected in surfaces water, at a concentration exceeding 1000 ng/L (Kasprzyk-Hordern et al., 2008; Rout et al., 2021). Recently, pesticides were reported in African surface water (K'oreje et al.,

2020). In their study, the concentrations of insecticides, herbicides, and fungicides were 0.06 ng/L-69 μg/L, 0.2 ng/L-14 μg/L, and 0.1 ng/L-9 μg/L, respectively.

EOPs in groundwater

Unlike surface water, the occurrence and concentrations of EOPs are lower in groundwater (Rout et al., 2021). The main contributors of EOPs to groundwater include landfill leachate, bank filtration, artificial recharge, percolation from agricultural land, and leakage from urban sewage systems, septic tanks, etc. (Lapworth et al., 2012; Luo et al., 2014; Stepien et al., 2013). In a national groundwater monitoring programme (Stuart et al., 2011), the British Geological Survey identified more than 1200 individual analytes of EOPs from around 3300 groundwater quality monitoring sites across England and Wales, and about 230 of these analytes were detected more than ten times from the groundwater samples. Noteworthily, the maximum concentrations of the most frequently detected EOPs, involving pesticides and their metabolites, chlorinated solvents and trihalomethanes, chlorinated solvents and trihalomethanes, ethylbenzene, PPCPs, and other organic contaminants, were found in the range of 500-8000 μg/L in the groundwater. Similarly, in a pan-European survey on the occurrence of 59 selected EOPs in European groundwater, Loos et al. (2010) collected and analyzed a total of 164 individual groundwater samples from 23 European countries, suggesting that the range of detection frequency for these EOPs were between 24% and 84% and considerably high concentrations of bisphenol A (2.3 μg/L), bentazone (32%; 11 μg/L), 1H-benzotriazole (1032 ng/L), terbuthylazine (716 ng/L), methylbenzotriazole (516 ng/L), desethylatrazine (487 ng/L), diethyltoluamide (454 ng/L), carbamazepine (390 ng/L), desethylterbutylazine (266 ng/L), atrazine (253 ng/L), caffeine (189 ng/L), perfluorooctanesulfonate (135 ng/L) and simazine (127 ng/L) were detected in groundwater samples. Karnjanapiboonwong et al. (2011) also reported the occurrence of steroid hormones, triclosan, caffeine, ibuprofen, and ciprofloxacin in groundwater samples at a land application site in the United States. But usually, the concentrations of EOPs detected in groundwater are at least an order of magnitude lower than in surface water (K'oreje et al., 2020; Rout et al., 2021).

EOPs in drinking water

Compared to groundwater and surface water, drinking water has the lowest concentration of EOPs, with a mean of 10 ng/L (Dos Santos et al., 2021). EOPs in drinking water mainly depends on the water sources and seasonal variations (Luo et al., 2014). In a previous study, Jiang et al. (2019) investigated the seasonal and spatial variations of 43 kinds of PPCPs in the water supply system of Changzhou in China, suggesting that the total concentrations of PPCPs in drinking water in urban areas were lower than in rural area. The health risk of PPCPs exposure through drinking water intake is relatively higher during summer than that in winter. Meanwhile, EOPs in drinking water could be related to the removal performance of drinking water treatment plants (DWTPs). Lin et al. (2016) reported the maximum concentrations of EOPs in the effluent of a DWTP located in the Taihu region was about 42.35 ng/L.

Similarly, Cai et al. (2015) found that the concentrations of EOPs in tap water from 6 DWTPs in Beijing were 1.8-38.5 ng/L. A recent study reported by Jiang et al. (2019) found that the total concentrations of detected PPCPs in drinking water range from 6.4ng/L to 809.3 ng/L, which is significantly higher than other reports in China. In another study carried out in Brazil, Reis et al. (2019) found that the less effective treatment process of the DWTPs leads to the high concentrations of EOPs in drinking water, with values of 6.32 μg/L for prednisone, 2.6 μg/L for betamethasone, and 0.56 μg/L for ketoprofen. In contrast, Valcárcel et al. (2011) reported that no traces of EOPs (24 selected pharmaceuticals) were detected in the drinking water of the Madrid Region in Spain. However, even the low concentrations of EOPs can be dangerous for human health through daily and long-term ingestion of contaminated drinking water (Wee et al., 2020).

Therefore, considering the variety of EOPs identified in the aquatic environment, including surface water, groundwater, and even drinking water, long-term studies should fruitfully explore the ecotoxicological risks of EOPs to the ecosystems and human health in the future.

2.1.2 Ecotoxicological effects and environmental risks of EOPs

The widespread existence of EOPs in aquatic environments is a growing concern due to their undesired synergistic effects (Rout et al., 2021). Although EOPs are generally found in the environment at trace concentrations ranging from ng/L to μg/L, the continuous discharge of EOPs and their metabolites into the environment from different pathways makes them "pseudo-persistent" (Lapworth et al., 2015; Mladenov et al., 2022). Consequently, many EOPs can persist in the aquatic environment and may bioaccumulate in organisms, which may cause serious ecotoxicological problems and pose extraordinary threats to ecosystems and human health (Dos Santos et al., 2021). Since the ecotoxicological assessments have the potential to evaluate the effect of EOPs at determined concentrations and different trophic levels, the environmental residue and potential ecotoxicity of EOPs have received increasing attention from scientists and the public (Köck-Schulmeyer et al., 2013; Wang et al., 2021; Zhong et al., 2021). Over the past decades, the ecotoxicological effects and environmental risks of EOPs have been increasingly addressed in the literature (**Fig. 2.2**).

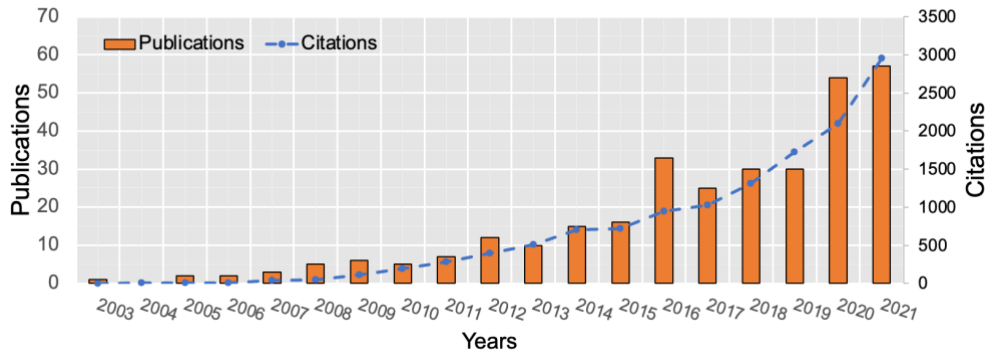

Fig. 2.2. Research concerns about the toxicology of EOPs in recent years. Data were collected from the web of science database (2003-2022, access date: 2022.3.01). Keywords: emerging organic contaminants or emerging organic pollutants or pharmaceuticals or personal care products and ecotoxicity.

The results of the ecotoxicological assessment are usually expressed as: 1) Average lethal concentration (LC_{50}): EOPs concentration that causes mortality of 50% of the organisms; 2) average effective concentration (EC_{50}): EOPs concentration that causes an acute effect on 50% of the organisms. In general, the risk levels of EOPs can be ranked as acute aquatic toxicity (> 100 mg/L, low concern; 1-100 mg/L, moderate concern; < 1 mg/L, high concern) and chronic toxicity (> 10 mg/L, low concern; 0.1-10 mg/L, moderate concern; < 0.1 mg/L, high concern) (European Commission, 2009). In this sense, numerous studies have been developed to assess the toxicity of EOPs in the aquatic environment and their impacts on the ecosystem. **Table 2.3** summarizes the common ecotoxicological effects for the evaluated EOPs. Potential concerns include endocrine disruption, reproductive damage, abnormal physiological processes, antibiotic-resistant bacteria, antibiotic resistance genes development, growth inhibition, increased cancer incidence, and other potential toxicity (Rout et al., 2021; Wang et al., 2021).

The toxicity of EOPs in the environment depends on the exposure time, pollutant concentration, type and stage of the organism, seasons and temperature, and synergistic effects of multiple compounds (Khan et al., 2020). For example, antibiotics in the environment can lead to the continuous selection for antibiotic resistant bacteria (ARB) that contain antibiotic resistance genes (ARGs), resulting in an increase in antibiotic tolerant microbial consortia and a threat to public health (García et al., 2020). In 2019, more than 2.8 million infections and 35000 deaths in the United States were caused by antibiotic-resistant bacteria and fungus (CDC, 2019). Moreover, an ecological risk assessment of 226 antibiotics to aquatic organisms indicated that more than 50% of antibiotics were a moderate concern (EC_{50} < 10 mg/L) for chronic toxicity to fish; 44% of antibiotics were a high concern (EC_{50} < 1 mg/L) for acute aquatic toxicity to *Daphnia*; 20% of antibiotics were a high concern (EC_{50} < 1 mg/L) for acute aquatic toxicity and 16% were a high concern (EC_{50} < 1 mg/L) for chronic toxicity to algae Sanderson et al. (2004). In another study, Isidori et al. (2007) tested the toxicity of antibiotics, including sulfamethoxazole, erythromycin, oxytetracycline, ofloxacin, and lincomycin, to aquatic organisms (e.g., bacteria, algae, rotifers, crustaceans, and fish), indicating that these EOPs could pose acute toxicity at the mg/L and cause chronic toxicity even at the μg/L level. Similarly, Kołodziejska et al. (2013) suggested that oxytetracycline could cause growth damage and reproductive reduction in crustaceans (*Daphnia magna*), duckweeds (*Lemna minor*), and algae (*Scenedesmus vacuolatus*). Zhang et al. (2015) reported that the occurrence of antibiotics in the Huangpu River could induce embryo mortality and morphological abnormalities in zebrafish (*Danio rerio*).

Table 2.3. Toxicological effects of typical EOPs.

Classification and compounds	Toxicological effects
Pharmaceuticals	
Antibiotics: Clarithromycin, Penicillin, Ofloxacin, Sulfonamides, Roxithromycin, and Tetracycline	Induce antibiotic resistance in microbial strains, alter microbial community structure, and cause a low population of algae, bacteria, nematodes, induce antibiotic-resistant bacteria and antibiotic resistance genes
Endocrine drugs: 17-β-estradiol (E2), estriol (E3), ethinyl estradiol (EE), 17-α-ethinylestradiol (EE2)	Interfere with the endocrine system, congenital disabilities, and developmental delays, affect reproduction and fertility, masculinization of females, feminization of males, bioaccumulation effect.
Nonsteroidal anti-inflammatory drugs: Diclofenac, Ibuprofen	Increased risk of gastrointestinal ulcers, kidney diseases, gill alterations of rainbow trout.
Lipid regulators: Clorfibric acid, and Gemfibrozil	Inhibition of bioluminescence, growth inhibition of microalgae.
Beta-blockers: Atenolol and Metoprolol	Affect reproduction and growth of fishes, inhibit receptor discharge in the gills
Anticonvulsants: Carbamazepine	Oxidation stress of rainbow trout, affects the central nervous system.
Personal care products	
Fragrances: Galaxolide, Musk xylene, Musk ketone	Toxic to aquatic organisms, cause oxidation stress to goldfish, carcinogenic to rodents, may damage the human nervous system
UV filters: Benzophenone-4, Oxybenzone, Benzophenone-3	Acute toxicity, affect growth and development, exhibit an estrogen effect, significant vitellogenin synthesis, decreases the percentage of hatched eggs
Industrial additives and agents	
Preservatives: Methyl paraben, 2-phenoxyethanol	Responsible for weak estrogenic activity
Fire retardants: Diethylstilbestrol	Affect brain and nervous system, hormone activity, reproduction and fertility, bioaccumulation effect.
Poly aromatic hydrocarbons: Anthracene, and Pyrene	Carcinogenic effect, cardiovascular diseases, poor fetal development, bioaccumulation effect.
Perfluorinated alkylated substances: Perfluorooctanoic acid	Thyroid disease, liver damage, kidney cancer, reduced response to vaccines, developmental effects on the unborn child.
Biocides	
Insect repellents: N, N-diethyl-3-methylbenzamide (DEET)	Caused no effects in lipid peroxidation levels nor on catalase activity, caused a significant reduction in carbohydrates levels

The information in this table is summarized from the recent reviews reported by Rout et al. (2021) and Wang et al. (2021).

The EOPs that might adversely affect the endocrine system are endocrine-disrupting compounds. These compounds are a group of highly heterogeneous chemical molecules, including 17-β-estradiol, estriol, ethinyl estradiol, and 17-α-ethinylestradiol, dichlorodiphenyltrichloroethane, organophosphate insecticides, genistein, phthalates, bisphenol A, polybrominated diphenyl ethers, diethylstilbestrol, etc. (Dos Santos et al., 2021; Wang et al., 2021). Depending on the time and dose of exposure, these EOPs can cause male and female sexual disorders (e.g., reduced male fertility, polycystic ovaries, higher miscarriage rates, longer time to conception, and altering both male and female gonad development) and increase cancer incidence (e.g., prostate, testicular, kidney, and breast cancers) (Oliveira et al., 2020; Stackelberg et al., 2004; Wang et al., 2021). For example, prolonged exposure to β-estradiol in μg/L and ng/L levels could induce vitellogenin in male Murray rainbowfish (*Melanotaenia fluviatilis*) and cause male feminization of an Indian frog (*Euphlyctis cyanophlyctis*) (Phuge and Gramapurohit, 2015; Woods and Kumar, 2011). Brion et al. (2004) observed that infertility occurs when fertilized zebrafish are exposed to 17-β-estradiol. A similar conclusion was reached by many studies (Armstrong et al., 2016; Kidd et al., 2007; Thrupp et al., 2018). This indicated that fertility decline is common in aquatic animals under the stress of endocrine-disrupting compounds (Wang et al., 2021).

In addition to the physiological toxicity to aquatic organisms, EOPs also have long-term bioaccumulation, which may also pose risks to ecosystems and humans (Langenbach, 2013; Langford et al., 2015; Navarro et al., 2016; Zarate et al., 2012). Generally, the commonly used EOPs, such as antibacterial agents (e.g., triclosan and triclocarban), bloody lipid regulators (e.g., gemfibrozil), anti-epileptic drugs (e.g., carbamazepine), β-blockers(e.g., propranolol), UV filters, biocides (e.g., DDT), perfluoroalkyl substances and halogenated flame retardants are often relatively highly bioaccumulated (Langenbach, 2013; Navarro et al., 2016; Wang et al., 2021). Because of these compounds' high lipophilicity and environmental stability, bioaccumulation occurs in organisms once released into the environment, followed by biomagnification in the food chain. Henriques et al. (2016) suggested that EOPs can be taken up by organisms from the surrounding environment and bioconcentrate several hundred folds, thereby posing a threat to ecosystems and human health. It is worth noting that the bioaccumulation of EOPs in wildlife has been reported in numerous studies (Balázs et al., 2016; Brausch and Rand, 2011; Grzesiuk et al., 2018; Thrupp et al., 2018), even in places with no local sources or industrial products such as the Arctic (Meador, 1996).

Therefore, the widespread usage and environmental persistence of the EOPs, alone or in mixtures, will have irreparable harmful effects, presumably leading to the

extinction of some ecosystems and posing a threat to human health. In this context, there is an urgent need to set stricter discharge limits for EOPs and develop advanced technologies for EOPs removal.

2.2 Constructed wetlands

In recent decades, numerous wastewater treatment technologies have been developed for EOPs removals, such as ozonation, Fenton oxidation, reverse osmosis, ionizing irradiation, membrane bioreactor, and other combined chemical and biological treatments (Deng, 2009; Wang and Wang, 2016). However, these technologies require a high level of energy consumption and are expensive to build and maintain, which are not entirely feasible for widespread application for small communities. Thus, there are growing appeals for developing more cost-effective treatment strategies to reduce the risk of EOPs into the ecosystems.

Constructed wetlands (CWs) are engineered systems that have been designed and constructed to utilize the natural processes, including substrates/supporting matrixes, wetland vegetation, and their associated microbial assemblages, to remove pollutants from contaminated water within a more controlled environment. CW development has received significant attention from scientists and the public, due to the lower cost, easy operation, and fewer maintenance requirements. In recent decades, the application of CWs has been significantly expanded to purify various types of wastewater, such as municipal, industrial and agricultural wastewater, mine wastewater, landfill leachate, stormwater, and agricultural runoff (Vymazal, 2011, 2009; Vymazal et al., 2021). Substrate, vegetation, and microbes are the main components in CWs, which play essential roles in wastewater purification. As shown in **Table 2.4**, pollutant removal in CWs is complex. It depends on various removal mechanisms, including the filtration and adsorption of the substrate, the uptake, fixation, transformation, and phytodegradation of wetland plants, and the decomposition, utilization, and dissimilation of microorganisms (Vymazal, 2014, 2011). Meanwhile, the removal efficiencies of pollutants in wastewater are affected by different types of CWs. Therefore, it is necessary to discuss the purification of pollutants in wastewater by various CWs.

2.2.1 Types of constructed wetlands

In general, the basic classification of CWs is based on the type of macrophytic growth, such as emergent plants (e.g., T*ypha domingensis*, *Phragmites australis*, *Scirpus Validus*, and *Iris pseudacorus*) (Vymazal, 2013), submerged plants (e.g., *Ceratophyllum demersum*, *Najas guadalupensis*, *Chara spp.* and *Potamogeton illinoensis*) (Dierberg et al., 2002), and free-floating plants (e.g., *Pistia stratiotes*, *Salvinia herzogii*, *Wolffia columbiana*, and *Lemna valdiviana*) (Herath and Vithanage, 2015). Further classification, as shown in **Fig. 2.3**, is usually based on the water flow regime (e.g., surface flow and sub-surface flow) and the water flow path in sub-surface wetlands (e.g., horizontal and vertical flow) (Vymazal, 2007). Different types of CWs can be combined (e.g., hybrid or combined systems) to utilize the specific advantages of the various strategies to enhance pollutant removal (Vymazal, 2011).

Table 2.4. Purification mechanisms of pollutants in CWs.

Purification mechanism	Pollutants	Position in CWs
Settlement	Suspended solids	Between aqueous phase and substrate
Filtration	Suspended solids	The substrate or plant interspace
Adsorption, ion exchange, chemical precipitation	Nutrients, emerging organic pollutants, and heavy metals	Substrates, plant root, and the attached biofilm
Microbial mineralization and transformation or biodegradation	Nutrients and emerging organic pollutants	Plant rhizome, substrate surface, biofilm, bottom sediment
Assimilation and uptake	Nutrients and heavy metals	Microbe, substrate, biofilm, and plant
Photodegradation/ Solar radiation	Emerging organic pollutants/ pathogenic bacteria	The surface of the wastewater
Predation	Pathogenic bacteria	Aqueous phase

Constructed wetlands with surface flow

Constructed wetlands with the surface flow (SFCWs) are a shallow sealed basin or sequence of basins with 20-30 cm of rooting soil and a water depth of 20-40 cm (Vymazal, 2010). SFCWs generally have a soil bottom and a water surface above the substrate (**Fig. 2.3**). The common wetland plants used in the FSCWs are emergent plants and floating plants. Dense emergent plants cover a considerable portion of the surface, usually more than 50% (Vymazal, 2010). Commonly, plants are not harvested in FSCWs, and the litter could provide organic carbon necessary for microbial activities such as denitrification, which may proceed in anaerobic pockets within the litter layer. Wastewater moves slowly through the SFCWs above the substrate. Consequently, the near-surface layer of water is aerobic, while the substrate and the deeper water are generally anaerobic (Halverson, 2004).

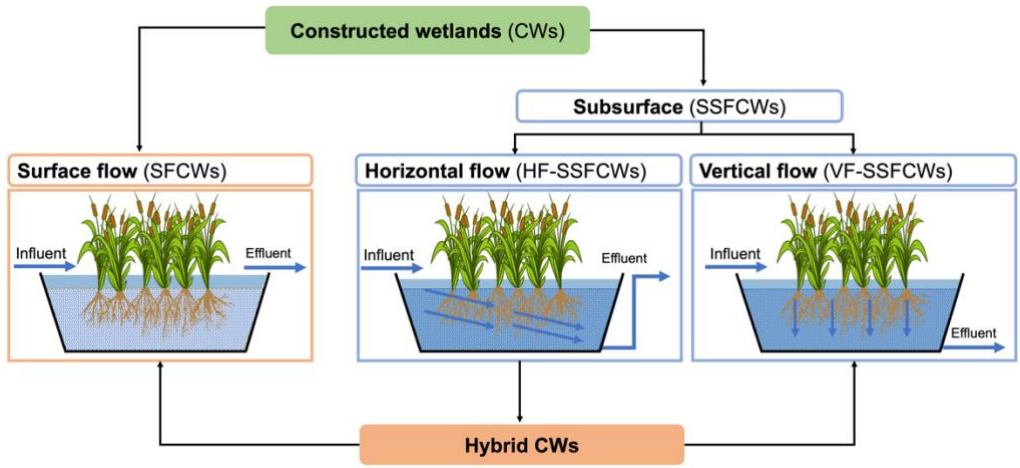

Fig. 2.3. Classification of constructed wetlands based on the water flow regime.

SFCWs are generally simpler to design and cheaper to construct than subsurface flow CWs (SSFCWs). They also provide a more diverse wildlife habitat because of areas of free water surface. Meanwhile, SFCWs offer greater flow control than SSFCWs, providing greater storm/surge capacity. The application of SFCWs can be very effective in removing organic matters and suspended solids through microbial degradation, filtration, and colloidal particle sedimentation (Vymazal, 2010). However, the main disadvantage of SFCWs is that they generally require more land than SSFCWs. In addition, odors and insects may be a problem due to the free water surface

of FSCWs, and the systems cannot operate appropriately in winter since the water surface is easy to be frozen (**Table 2.5**). Therefore, SFCWs are commonly used for tertiary treatment of municipal wastewater, mine drainage waters, and stormwater runoff.

Constructed wetlands with subsurface flow

Subsurface flow CWs (SSFCWs) are generally constructed with a porous material, such as soil, sand, or gravel, as a substrate for growing wetland plants and developing various microbes. In these systems, the bed depth is usually less than 0.6 m, and typical flow depths range from 0.49 to 0.79 m (Halverson, 2004). The wastewater flows slowly through the substrate under the bed's surface planted with vegetations. The emergent plants, mostly reeds, bulrush, and sometimes cattails, are the main wetland plant species in SSFCWs due to their large biomass, well-developed root system, strong capacity of oxygen transport, and strong pollution resistance (Shelef et al., 2013). Therefore, SSFCWs are also called “Reed beds” in Europe, “Reed bed treatment system” in the United Kingdom, and “vegetated submerged beds” because common reed (*Phragmites australis*) is frequently planted in this systems (Vymazal, 2011).

The application of SSFCWs is thought to have more advantages than that of SFCWs (**Table 2.5**). For example, the substrate in SSFCWs provides more surface area for the growth of bacterial biofilm than SFCWs, thus resulting in a higher rate of pollutant removal per unit of land than SFCWs (Halverson, 2004). Since the wastewater is generally kept below the ground surface, SSFCWs have little risk of odors emission and insect overpopulation (e.g., mosquitoes and flies) and minimal risk of animal or public exposure or contact with the wastewater. In addition, SSFCWs are more suitable for cold-weather operation than SFCWs due to the insulation of the accumulated plant debris on the SSFCW surface. However, SSFCWs are suitable for treating wastewater with relatively low solids concentration to prevent plugging of the substrate (Vymazal, 2010).

Table 2.5: Advantages and disadvantages of surface and subsurface flow constructed wetlands.

CW types	Advantages	Disadvantages
SFCWs	Conventional treatment methods, simple design, less investment to construct and operate, greater flow control, used for higher suspended solids wastewater, and more diverse wildlife habitat.	Lower contaminant removal rates required more land, risk of ecological or human exposure to surface-flowing wastewater, easy freeze in winter, easy breed flies and other insects, and odor emission.
SSFCWs	Higher rates of contaminant removal required less land, more accessible maintenance, good hygienic conditions, and abundant microorganism.	High infrastructure requirements, high investment cost, and easy to plug when treating sewage with high suspended solids

Sources: Halverson (2004).

In SSFCWs, wastewater flows horizontally or vertically through the substrate and below the ground surface so that they can be classified into two basic flow systems: horizontal flow (HF-SSFCWs) and vertical flow (VF-SSFCWs) (**Fig. 2.3**). Horizontal and vertical flow systems have similar pollutant removal mechanisms, and both of them are very effective in removing contaminants and suspended solids. However, contrary to HF-SSFCWs, VF-SSFCWs are fed with intermittent hydraulic loading (Vymazal, 2011). Therefore, the exchange of wet and dry periods provides more significant oxygen transportation into the bed, thus promoting nitrification and aerobic biodegradation (Vymazal, 2007). Due to the horizontal flow, the influence of the substrate on the interception, filtration, and adsorption of pollutants is limited in HF-SSFCWs. VF-SFFCWs, by comparison, require less land and are more effective in the removal of contaminants (e.g., organic matters, heavy metals, and emerging organic pollutants). For these reasons, VF-SFFCWs are usually used for primary or secondary treatment, while HF-SFCWs are often used to treat wastewater diluted with stormwater runoff (Gorgoglione and Torretta, 2018; Vymazal, 2010).

Hybrid constructed wetlands

Various types of CWs could be combined to achieve higher removal efficiency by using the advantages of individual CW systems (Vymazal, 2011). At present, hybrid CWs are in operation in many countries worldwide (Vymazal, 2011). In a survey of 60 hybrid constructed wetlands from 24 countries reported between 2003 and 2013, Vymazal (2013) suggested the most commonly used hybrid system is a VF-HF constructed wetland applied to treat both sewage and industrial wastewater. HF-VF hybrid systems have also been reported to treat municipal wastewater. Meanwhile, SFCWs are also used in several hybrid configurations (Torrijos et al., 2016; Vymazal, 2013). Moreover, the hybrid systems consist of multistage (e.g., VF-VF-HF, VF-HF-VF, SF- SF-HF, VF-VF-HF-VF, or VF-VF-VF-HF-VF have been used for the treatment of sewage and several kinds of industrial wastewater (Vymazal, 2013). In general, the hybrid CWs efficiently remove pollutants, especially for total nitrogen (Vymazal and Kröpfelová, 2015). For these reasons, hybrid CWs with different configurations have been used to treat a variety of wastewater, including domestic or municipal sewage (Yan et al., 2021), landfill leachate (Bakhshoodeh et al., 2020), compost leaching, agricultural runoff (Wang et al., 2018b), slaughterhouse, shrimp and fish aquaculture (Vymazal, 2013), winery, and other industrials (Vymazal, 2014).

2.2.2 Removal of EOPs in CWs

Owing to the unique advantages of easy operation and maintenance, eco-friendliness, and low operational and maintenance costs as well as low energy input, CWs have been considered to be a sustainable technology for removing or attenuating a variety of waterborne contaminants, including EOPs (Ávila et al., 2013; Vymazal, 2014, 2010; Vymazal et al., 2021). The first attempts to investigate the performance of CWs in removing EOPs were reported in Portugal (Dordio et al., 2007), Spain (Matamoros et al., 2007), the United States (Conkle et al., 2008), Korea (Park et al., 2009), Denmark (Matamoros et al., 2009), or Italy (Ranieri et al., 2011). After that, more and more studies have been devoted to concerning the removal of EOPs in CWs, and most of them are summarized in reviews conducted by Zhang et al. (2014), Carvalho et al. (2014), Dhir (2019), García et al. (2020), and Ji et al. (2022). In a previous, Vymazal et al. (2017) evaluated the occurrence and removal of 31 different EOPs (e.g., antibiotics, antiepileptics, antiphlogistics, antibacterials, anticoagulants,

contrast mediums, etc.) in four full-scale CWs located in rural areas of the Czech Republic, indicating that CWs could be a useful tool in removing EOPs from municipal wastewater. Several studies have documented that the application of CWs as tertiary treatment systems has provided a comparable removal efficiency for EOPs to advanced treatment systems (Conkle et al., 2008; Matamoros and Salvadó, 2012; Zhang et al., 2014a).

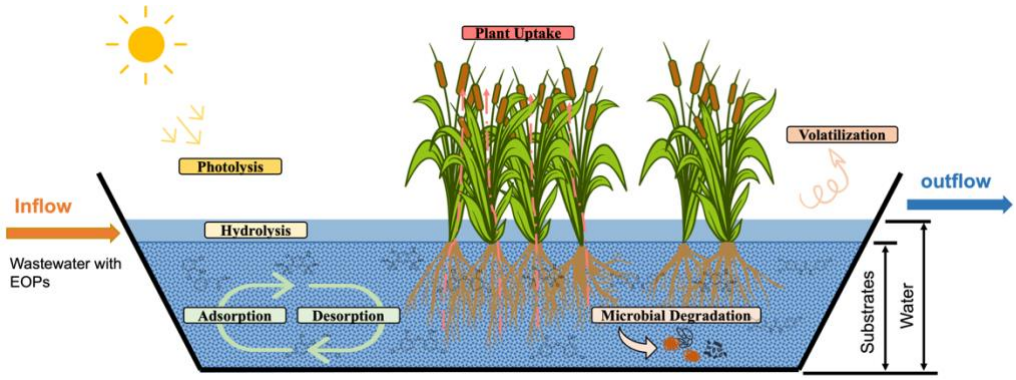

Fig. 2.4. EOPs (emerging organic pollutants) removal mechanisms in constructed wetlands.

The high removal efficiencies of EOPs in CWs can be explained by the combination and synergy of various physical, chemical, and biological removal mechanisms (Vymazal et al., 2017; Zhang et al., 2014a). A general EOPs removal mechanism in CWs is shown in **Fig. 2.4**, which involves various processes, including photodegradation, hydrolysis, volatilization, plant uptake, phytodegradation, microbial degradation, and adsorption/desorption by the substrate. Among them, the dominant removal mechanism in CWs is adsorption to the substrate and/or sorption to organic surfaces, followed by microbial degradation (e.g., aerobic and anaerobic), plant uptake (planted CWs), and photodegradation (Ilyas and van Hullebusch, 2020). Meanwhile, the removal processes of EOPs in CWs may also be influenced by their physiochemical properties, such as Log K_{oc}, Log K_{ow}, Log D_{ow}, cationic or anionic nature (pKa), solubility in water, molecular weight/structure, and presence of certain elements (e.g., Cl and Br) (Ilyas and van Hullebusch, 2020; Zhang et al., 2014a). Therefore, the removal efficiency of different EOPs varies among the different types of CWs.

However, EOPs and their metabolites in CW systems may also cause adverse effects on the stability of the system operation (Moro et al., 2014). For example, a

previous study reported by Stevens et al. (2009) suggests that triclosan could influence the growth of wetland plants (e.g., seed germination and root development) even at a low concentration (0.6 μg/L). Similarly, Ziółkowska et al. (2014) observed the adverse effects of EOPs on leguminous plants (lupin, pea and lentil), such as reducing seeding growth and change in enzymatic activity. In addition, Some EOPs are chronically toxic to animals (e.g., *Daphnia*), microalgae, and microorganisms (e.g., bacteria and fungi) (Breitholtz et al., 2012; Crane et al., 2006; Rathi et al., 2021). Therefore, there is an urgent need to eliminate the adverse impacts of EOPs on the stability of wetland systems, which is beneficial for the removal of EOPs in CWs.

2.2.3 Effect of CW design parameters on EOPs removal

Effect of CWs configuration

In general, the physicochemical parameters (e.g., dissolved oxygen (DO), pH, oxidation-reduction potential (ORP), and electrical conductance) in CWs are strongly influenced by the type of CWs (Matamoros and Bayona, 2006; Zhang et al., 2014a); thus CWs differ greatly in configuration, including individual systems (e.g., SFCWs and SSFCWs, HF-SSFCWs and VF-SSFCWs) and hybrid systems, along with varying kinds and depth of substrate.

Ilyas et al. (2021) investigated the removal of 59 EOPs, including 33 pharmaceuticals, 15 personal care products, and 11 steroidal hormones, in CWs. They found that some of the selected EOPs, such as oxytetracycline, acetaminophen, sulfadiazine, and triclosan, showed better removal efficiency in FSCWs; the HF-SSFCWs had better removal efficiency of ofloxacin, 17βestradiol, and estrone, while the VF-SSFCWs showed better performance for the removal of clarithromycin, ibuprofen, naproxen, salicylic acid, estriol, and testosterone; at last, the hybrid CWs performed a better removal efficiency of 17α-ethinylestradiol, diclofenac, erythromycin, gemfibrozil, sulfamethazine, and sulfamethoxazole.

Photodegradation would be responsible for the major removal mechanism of EOPs in SFCWs, although both plant uptake and biodegradation also contribute to the overall performance of the systems (Carlos et al., 2012; Zhang et al., 2014a). Photodegradation has been described as the most important and efficient removal

pathway for some certain EOPs and could affect the environmental persistence of EOCs in surface water (Yan et al., 2015). In aquatic systems, the efficiency of photodegradation for EOPs removal depends on several factors, including the absorbance spectrum of EOPs, sunlight availability for photolysis, the quantum yield of photolysis, light intensity and light attenuation by water depth (Buser et al., 1998; Klavarioti et al., 2009). Therefore, SFCWs show better performance when compared to SSFCWs for EOPs, which are sensitive to photodegradation because this process may take place only in unplanted FSCWs or ponds where water is directly exposed to sunlight (Reyes-Contreras et al., 2012). For example, triclosan and diclofenac have great potential for photodegradation, and their removal in FSCWs was significantly higher than that in SSFCWs (Ilyas et al., 2021; Zhang et al., 2014a).

In SSFCWs, due to the prevailing anaerobic and aerobic conditions, the corresponding biodegradation is obvious for eliminating EOPs besides their removal by plant uptake and substrate (e.g., sedimentation, and adsorption/desorption). Compared with SFCWs, SSFCWs have more adsorption surfaces and superior rhizosphere effects, which could provide a better growth environment for microbes, resulting in a higher diversity and greater activities of microorganisms (Zhang et al., 2014a). Thus, the EOPs removal performance of SSFCWs is typically better than that of SFCWs (Ilyas et al., 2021). Meanwhile, VF-SSFCW is mainly aerobic, which is more conducive to the aerobic biodegradation of EOPs compared with HF-SSFCW (Ávila et al., 2014; Matamoros et al., 2012). Therefore, VF-SSFCWs exhibit a higher removal capacity of EOPs than HF-SSFCW. In a previous study, Matamoros et al. (2007) found that the better removal of the selected EOPs, such as ibuprofen, diclofenac, naproxen, salicylic acid, and caffeine, was observed in VF-SSFCWs compared to HF-SSFCW.

Moreover, as mentioned above, hybrid CWs can exploit individual systems' specific advantages by combining various CWs to achieve a better pollutant removal performance. Some studies suggested that the removal efficiency of EOPs could be enhanced by the combination of anaerobic and aerobic biodegradation (Ilyas et al., 2021; Zhang et al., 2014a). Therefore, individual CWs may not achieve the high removal efficiency of EOPs because they cannot provide both aerobic and anaerobic conditions simultaneously. Several studies revealed that the removal efficiency of EOPs in hybrid CWs was higher than that in VF-SSFCW and HF-SSFCW, mainly due to the coexistence of aerobic and anaerobic conditions in hybrid CWs (Ilyas and van Hullebusch, 2020; Kahl et al., 2017; Zhang et al., 2014a).

Overall, the decision on the suitable type of CWs, such as individual systems and hybrid systems, can be made based on the performance of CWs for the removal efficiency of EOPs. In the present study, the performance of CWs in removing EOPs was only considered in the VF-SSFCWs with emergent plants.

Effects of plants

A large variety of plants, for instance, such as *Phragmites australis*, *Typha angustifolia*, *Typha latifolia*, *Iris pseudacorus*, *Scirpus validus*, *Glyceria maxima*, *Salix alba*, *Spirodela polyrhiza*, *Cyperus alternifolius*, and *Thalia dealbata*, were used in CWs for the removal of EOPs (Ilyas et al., 2021; Vymazal et al., 2017). The selection of plant species was depended upon the availability of plants in different climatic regions. Commonly, a native wetland plant species is the best candidate, and the most widely used species include *Phragmites australis*, *Typha angustifolia* (Ilyas et al., 2021).

The presence of plant seem to improve the removal efficiency of EOPs due to the stimulatory effects of plants, such as insulation against low temperatures, facilitating the release of oxygen and root exudate in the rhizosphere, thus providing a favorable condition for the growth of microorganisms and promoting aerobic biodegradation (Ilyas et al., 2021). Therefore, wetland plants play a crucial role in removing EOPs in CWs. Numerous studies have shown that the removal efficiency of EOPs can be significantly enhanced in planted CW systems compared to unplanted CW systems (Hijosa-Valsero et al., 2010; Ilyas et al., 2021; Zhang et al., 2014a). For example, the removal efficiency of pharmaceuticals (e.g., ibuprofen, diclofenac, naproxen, salicylic acid, caffeine, carbamazepine, gemfibrozil, and sulfamethoxazole,), personal care products (e.g., triclosan, galaxolide, methyl dihydrojasmonate, and tonalide), and steroid hormones (e.g., 17α-ethinylestradiol) in planted CWs were higher than that in unplanted CWs (Ilyas and van Hullebusch, 2020).

Meanwhile, plant uptake and bioaccumulation in plant tissues have been considered as one of several major removal pathways for EOPs in CWs (Vymazal et al., 2017; Zhang et al., 2014a). In recent decades, more than 100 EOPs have been proven to be uptaken by plant roots (Carvalho et al., 2014; Ravichandran and Philip, 2021; Wu et al., 2015a). For example, Petrie et al. (2018) observed the uptake of methylparaben by plant roots (*Phragmites australis*) and the bioaccumulation of

methylparaben in both aboveground (106-246 μg/kg) and underground (152 μg/kg) parts of the plant. Diffusion processes generally drive the uptake and transport of EOPs within plant tissues due to the lack of specific transporter proteins (Dhir, 2019). It has been found that this process can be influenced by the physiochemical properties of EOPs, such as the dissociation constant (pKa), octanol-water partion coefficient (K_{ow}), and solubility (Dordio and Carvalho, 2013; Miller et al., 2016). Recently, Abril et al. (2021) reported the uptake, bioaccumulation and translocation of 22 different EOPs from various chemical classes (such as surfactants, plasticizer, preservatives, perfluorinated compounds, biocides, and UV filters) in plants (*Raphanus sativus*), and proven that the distribution of EOPs in plant tissues varies with their physicochemical properties.

Following plant uptake, EOPs may undergo partial or complete degradation, or they may be metabolized or transformed to less toxic compounds and bound in plant tissues in unavailable forms (Zhang et al., 2014a). Some studies reported that wetland plants could metabolize EOPs through phytodegradation (Miller et al., 2016; Ravichandran and Philip, 2021). In the processes of phytodegradation, various enzymes in plant tissues, such as cytochrome P450 monooxygenases, glycosyltransferase, glutathione-S transferase, peroxidases, hydrolases, etc., may act on EOPs and mineralize them either completely into inorganic compounds or partially into metabolic products that are stored in plant tissues (Bartha et al., 2014; He et al., 2017). He et al. (2017) explored ibuprofen uptake and transformation in *Phragmites australis*. They observed that four intermediates were identified in plant tissues, including hydroxy-ibuprofen, 1,2-dihydroxy-ibuprofen, carboxy-ibuprofen, and glucopyranosyloxy-hydroxy-ibuprofen. They hypothesized that cytochrome P450 monooxygenases first catalyzed the transformation of ibuprofen in plant tissues, and then by glycosyltransferase, followed by further storage or metabolism in vacuoles or cell walls. Bartha et al. (2014) also reported the metabolism of diclofenac in the wetland plant species *Typha latifolia*, and detected parent diclofenac, hydroxy-diclofenac, glucopyranosyloxy-hydroxy-diclofenac, and diclofenac-glutathione conjugate in roots, and only diclofenac and hydroxy-diclofenac in leaves. In a recent study, Ravichandran and Philip (2021) investigated the uptake and fate of tree different EOPs (atenolol, carbamazepine, and diclofenac) in two wetland plant species (*Canna indica* and *Chrysopogon zizanioides*) and found that the accumulated parent EOPs concentrations in plant tissues were relatively low (< 1%), with a larger fraction getting metabolized (43.9-81.8%). Metabolites such as atenololic acid, seven transformation products of

carbamazepine and six metabolites of diclofenac were detected in both of these two plant species.

Effects of substrate

Substrate, also known as media, filling material, support matrix/material, is one of the major components in CWs, which have been widely acknowledged to play a critical role in CWs, such as providing a carrier for plant growth and biofilm development and removing pollutants through absorption and adsorption (Wu et al., 2015b; Yang et al., 2018). Based on the material of substrates, they can be divided into two categories: conventional substrates (e.g., sand, soil, and gravel) and emerging substrates (e.g., construction wastes, tire chips, zeolite, perlite, vermiculite, biochar, etc.) (Yang et al., 2018). An appropriate selection of substrates is an essential aspect in the optimization of CWs as the interactions in the micro-environment of CWs directly influence the performance and efficiency of pollutant removal, especially for the removal of EOPs (Dordio and Carvalho, 2013; Li et al., 2014) and toxic metals (Cheng et al., 2002; Yang et al., 2018).

The role of substrate in the removal of EOPs has been investigated by using different types of substrate materials, such as high adsorption capacity, porous structure, rich in organic/inorganic surfaces, high surface area, etc., suggesting that adsorption to the substrate and/or sorption onto organic/inorganic surfaces is a vital removal mechanism to eliminate EOPs in CWs (Chen et al., 2016a; Ilyas and Van Hullebusch, 2020; Ji et al., 2022). Adsorption of EOPs onto the substrate surface involves different mechanisms such as electrostatic interactions, surface complexation, ion exchange, hydrophobic partitioning, and van der Waals interactions (Dordio and Carvalho, 2013; Li et al., 2014). Polar or ionic EOPs are predominantly adsorbed onto substrate (e.g., light expanded clay aggregate, biochar, vermiculite, and zeolite) through electrostatic interactions or ion exchange. In contrast, hydrophobic processes can preferentially adsorb non-polar EOPs onto a particularly organic-rich substrate such as rice straw, oyster shell, compost, biochar, and organic wood-mulch (Li et al., 2014; Yang et al., 2018). Meanwhile, substrates rich in organic matter can provide the necessary carbon source to promote microbial processes and substrate with porous structure and/or larger surface area could promote the development of biofilms and increase the contact area with contaminants in wastewater. Therefore, the substrate may influence the removal of EOPs by affecting the microbial community structures in CW. In a previous study,

Li et al. (2010) investigated microbial community structure in eight substrates, zeolites, anthracite, shale, vermiculite, ceramic filter media, gravel, steel slag, and bio-ceramic. They found that the microbial community composition showed significant differences among the substrates. Similarly, Zhang et al. (2018) found that although the adsorption capacities of the selected substrates (e.g., sand, zeolite, blast iron slag, petcock, polonite, and crushed autoclaved aerated concrete) are low, the type of substrates influences the microbial community metabolic function not only in the biofilm but also in the interstitial water. These indicated that substrate could be a driver to enhance EOPs removal by adsorption and altering microbial community function in CWs.

2.3 Arbuscular mycorrhizal fungi

Arbuscular mycorrhizal fungi (AMF), as one of the commonly occurring heterogeneous groups of the biological organism in the natural environment, can form a mutualistic symbiosis with a broad range of plants (about 80 to 90%), such as tropical forests, grasslands, alpine and croplands (Smith et al., 2010; James M. Trappe, 1987). According to the confirmation of DNA sequence data, Redecker et al. (2000) found that AMF has revolutionized into living fossils in 460 million years by benefiting the host plant. The plant-mycorrhizal association can develop the exraradical mycelium beyond the root-hair zone and establish tree-shaped subcellular structures within root cells, thus, improving nutrient acquisition and plant tolerance to environmental stresses, including drought, cold, salinity, heavy metals, and organic contaminants (Ajit Varma, 2017; Hu et al., 2020a). In addition to providing these benefits for the host plants, arbuscular mycorrhizal (AM) symbiosis also plays a significant role in boosting the development of rhizosphere microorganisms by stimulating the production of root exudates, phytoalexins and phenolic compounds (Latef et al., 2016; Toljander et al., 2007). For these reasons, AMF has been regarded as a critical phytoremediation approach to re-establish the degraded ecosystems, such as contaminated soils, abandoned agricultural fields and grassland (Ajit Varma, 2017).

2.3.1 AMF in wetland ecosystems

Occurrence of AMF in wetlands

The symbiotic relationship between AMF and plant roots in wetland ecosystems has kindled little research interest in the past. Compared to terrestrial plants, wetland plants are usually rooted in water-saturated or even flooded substrates, which frequently results in anoxic conditions and thus limits the survival of AMF and the establishment of symbiosis (Huang et al., 2021). In recent decades, the occurrence and functional roles of AMF in wetland ecosystems have gradually attracted attention (**Fig. 2.5**). Numerous studies have shown that AMF is relatively common in wetland ecosystems, the symbiotic relationships between AMF and plant roots have been found from various wetland habitats, including fens, swamps, marshes, shorelines, bays, floating wetland mats, and natural and constructed wetlands (Fusconi and Mucciarelli, 2018; Huang et al., 2021; Ramírez-Viga et al., 2020). In a previous review, Xu et al. (2016) summarized the mycorrhizal status in wetland habitats, suggesting that the symbiotic relationship between AMF and plant roots has been found in 99 families of wetland plants living in 31 different habitats, even including submerged aquatic plants and several plant species that were thought to be nonmycorrhizal (e.g., *Cyperaceae*, *Chenopodiaceae*, and *Plumbaginaceae*). **Table 2.6** summarizes the AMF colonization of plant species (partly) observed in aquatic habitats.

The occurrence of AMF in wetland ecosystems is possible according to two main scenarios: 1) Some fungi can tolerate hypoxic conditions; 2) Plant roots could provide the fungi with sufficient oxygen (Miller et al., 1999). Neto et al. (2006) found that once AMF has already colonized the roots, their abundance can be maintained under flooded conditions and, in some cases, even increased. A similar phenomenon also was observed for the number of spores from AMF in flooded soil (Miller and Sharitz, 2000). Meanwhile, the potential for AMF colonization of wetland plants can be increased with their well-developed aerenchyma, which can facilitate effective gas exchange between the atmosphere and wetland environment (Gaberščik et al., 2017). For example, Nielsen et al. (2004) reported that the extensive aerenchyma of the wetland plant genus *Typha* was very effective of aeration in the rhizosphere and promoted AMF during flooding conditions. The major survival strategies of AMF in wetland ecosystems are shown in **Fig. 2.6**.

In addition, the percentages of mycorrhizal status in the roots of wetland plants show differences under the various wetland habitats. This difference results from multiple factors, such as the type and size of wetlands, wetland plant species, climate, hydraulic conditions, available nutrients, water quality (e.g., contaminant sources, types, and concentrations), and fungi species. Choudhury et al. (2010) investigated mycorrhizal status in plant roots grown in the marshy and shoreline. They found that the predominant fungal species in the rhizosphere soil samples were *Glomus morphotypes*, and a total of 18 different inoculated plant species were recorded to be composed of four genera, including *Glomus* (66.67%), *Acaulospora* (16.66%), *Gigaspora* (11.11%), and *Scutellospora* (5.56%). Wu et al. (2014) observed the differences in the mycorrhizal status of *Funnelliformis mosseae* (16.5%) and *Rhizophagus irregularis* (18.1%) in the roots of *Phragmites australis* under the same experimental conditions. According to the review reported by Xu et al. (2016), mycorrhizal status in monocots (13%) is generally lower than that in dicots (58%). Commonly, AMF colonization in the roots of wetland plants is invariably more ignoble than that of terrestrial plants (Lumini et al., 2011). However, the presence of AMF has a wide range of benefits for their host plants.

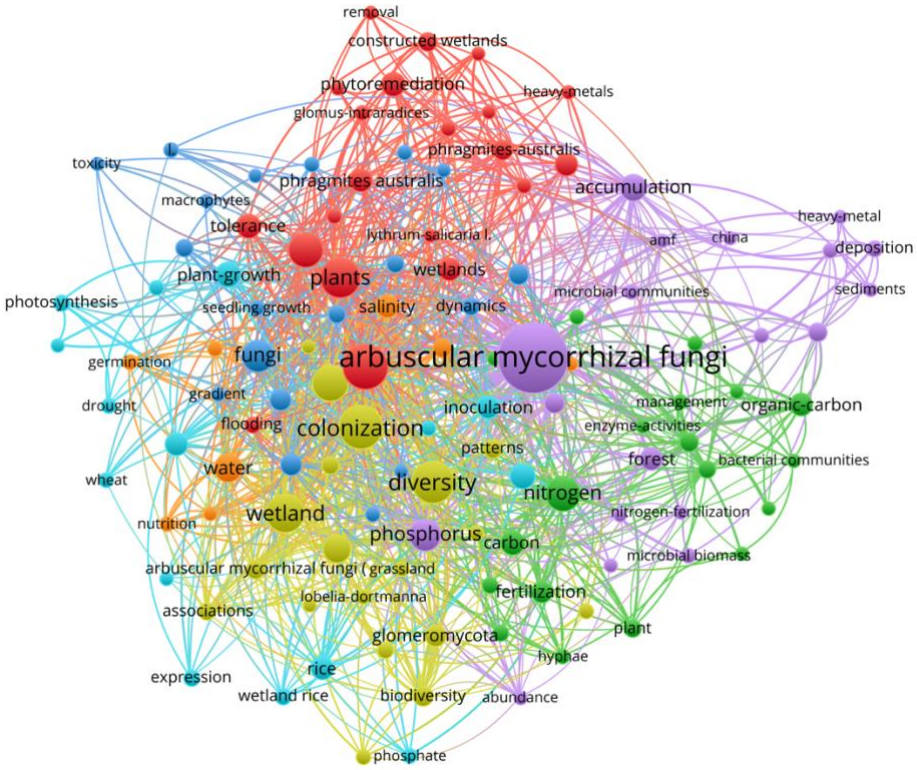

Fig. 2.5. Mapping on the co-occurrence of keywords related to arbuscular mycorrhizal fungi in wetlands. Data were collected from the Web of Science Database (184 research papers, access date: 2022.3.20). Keywords: wetlands and arbuscular or arbuscular mycorrhizal fungi. The colors represent clusters of extracted terms grouped by the software (VOSviewer 1.6.18) according to the items relations; the size of the circle reflects the keywords recurrence; the weight of lines between circles demonstrates the intensity of keywords co-occurrence.

Table 2.6. AMF colonization in the roots of plant species observed in wetland habitats.

Plant species (family)	Habitats (location)
Acanthus ilicifolius Linn (Acanthaceae)	Mangrove (China)
Dicliptera brachiata (Pursh) Spreng. (Acanthaceae)	Bottomland hardwood forest (USA)
Acer rubrum L. (Aceraceae)	Calcareous fen, freshwater wetland (USA)
Sesuvium portulacastrum (Aizoaceae)	Mangrove
Centella asiatica (L.) Urb. (Apiaceae)	Marshy (India)
Ilex verticallata (L.) Gray (Aquifoliaceae)	Freshwater wetland (USA)
Artemisia annua L. (Asteracea)	Bay (China)
Sagittaria latifolia Willd. (Asteraceae)	Freshwater marsh (USA)
Symphyotrichum subulatum (Michx.) GL Nesom (Asteraceae)	Degraded cypress swamp (USA)
Impatiens pallida (Balsaminaceae)	Lake Erie (USA)
Lobellia perpusilla (Campanulaceae)	Lake (New Zealand)
Inula crithmoides L. (Cupressaceae)	Saltmarsh (Portugal, Spain)
Kalmia latifolia L. (Ericaceae)	Freshwater wetland (USA)
Pterocarpus officinalis (Jacq.) (Fabaceae)	Pterocarpus officinalis (Jacq.) (Fabaceae)
Geranium robertanum (Geraniaceae)	Lake Erie (USA)
Geranium maculatum L. (Geraniaceae)	Freshwater wetland (USA)
Proserpinaca palustris L. (Haloragaceae)	Freshwater wetland (USA)
Myriophyllum pedunculatum (Haloragaceae)	Lake (New Zealand)
Iris versicolor L. (Iridaceae)	Calcareous fen (USA)
Juncus gerardi (Juncaceae)	Coastal salt marsh (USA)
Schizachyrium scoparium Michx. Nash (Poaceae)	Freshwater wetland (USA)
Glyceria plicata (Poaceae)	Grassland, scrub, woodland (UK)

Plant species (family)	Habitats (location)
Ixeris polycephala Cass. (Poaceae)	Freshwater marshland (China)
Paspalum dilatatum (Poaceae)	Subtropical aquatic and marshy
Phragmites australis (Cav.) Trin. ex Steude (Poaceae)	Oligotrophic wetland, constructed wetland, wetland (Denmark, Germany, USA)
Phragmites communis Trin. (Poaceae)	Bay (China)
Typha latifolia L. (Typhaceae)	Prairie fen, freshwater wetland, floating wetland mat, experimental wetland, wetland, floating (USA)
Typha glauca Godr. (Typhaceae)	Floating wetland mat (USA)
Typha angustifolia L. (Typhaceae)	Floating wetland mat, wetland (USA)
Typha australis (Typhaceae)	Lake Retba (Senegal)
Geum rivale L. (Rosaceae)	Calcareous fen (USA)
Galium rotundifolium (Rubiaceae)	Subtropical aquatic and marshy
Azolla pinnata L. (Salviniaceae)	Marshy and shoreline, free-floating (India)
Leucospora multifida (Michx.) Nutt. (Scrophulariaceae)	Bottomland hardwood forest (USA)
Physalis longifolia Nutt. (Solanaceae)	Bottomland hardwood forest (USA)
Litorella uniflora (Plantaginaceae)	Bay (China)
Littorella uniflora (L.) Asch. (Plantaginaceae)	Oligotrophic wetland (Denmark)
Limonium sinense (Girard) Kuntze (Plumbaginaceae)	Bay (China)
Limonium caralinianum (Plumbaginaceae)	Coastal salt marsh (USA)

Information is collected by Xu et al. (2016).

Effects of AMF on wetland plants

Similar to terrestrial ecosystems, AMF also exhibits many beneficial effects on wetland plants in wetland ecosystems, such as plant growth and root morphological improvement, enhancement of antioxidant defense systems, induction of oxidative damages, and increased tolerance for abiotic stresses. The main positive effects of AMF on wetland plants are summarized in **Fig. 2.6**.

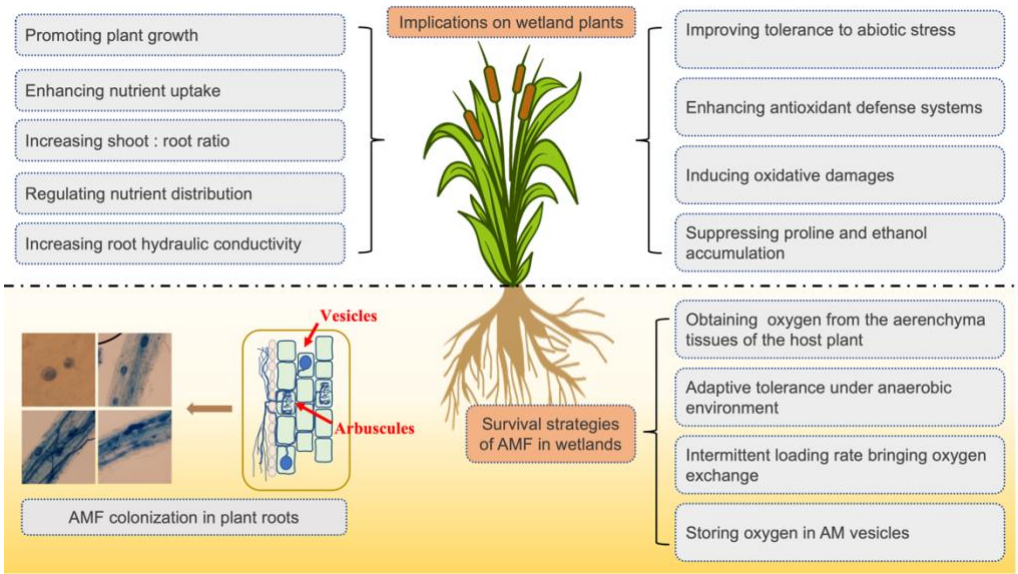

Fig. 2.6. A schematic diagram displays various survival strategies of AM fungi adapted to the wetland environment and their positive implications on wetland plants.

An important function of AMF in wetland ecosystems is to promote the growth of the host plant under various environmental stresses. Previous studies found that AMF inoculation could improve plant growth and root morphology under waterlogging (Huang et al., 2021). Zheng et al. (2020) observed that the root morphological properties (e.g., root length, root surface area, and root volume) of *Prunes persica* were improved by AMF under 12 days of waterlogging. Similarly, the improvement of plant growth and root morphology by AMF inoculation was highlighted in *Phalaris arundinacea* and *Scirpus sylvaticus* , *Citrus junos*, *Carex tribuloides*, and *Rumex orbiculatus* (Fraser and Feinstein, 2005; Hu et al., 2020b; Wu et al., 2013). The improvement of inoculated plants in growth under flooding conditions could be

originated from AMF-induced osmotic adjustment and the enhancement of nutrient uptake (e.g., N, P, K, and some micronutrients) (Huang et al., 2021; Xu et al., 2016). In addition, AMF enhance the host plant's antioxidant defense systems to maintain normal and protect plant cells from any oxidative damage (Hu et al., 2020a; Wu et al., 2013). Hu et al. (2020a) found that the wetland plant (*Iris wilsonii*) inoculated with *Rhizophagus irregularis* recorded higher antioxidant responses (vis an increased activity of superoxide dismutase and peroxidase) than non-inoculated plant under Cr stress. In a previous study, Lenoir et al. (2016) revealed that 42 putative genes in the fungi of *Rhizophagus irregularis* were identified as a part of the antioxidant system, suggesting that AMF has its antioxidant defense systems to respond against various abiotic stresses. In addition, AMF can protect the host plant from damages by influencing the transport and distribution of contaminants in plant tissues. Previous studies found that AMF could reduce the concentration of heavy metals and EOPs in plant shoots by promoting their bioaccumulation in plant roots (Debiane et al., 2009; Ferrol et al., 2016; Langer et al., 2010). In aquatic habitats, AMF has the potential to influence the composition, succession, and diversity of wetland plant communities (Weishampel and Bedford, 2006). Xu et al. (2016) suggested that AMF mainly affects plant diversity by regulating plant competition and affecting community uniformity, thus contributing to building or re-establishing plant communities in contaminated wetland ecosystems.

Therefore, AMF may have an essential function in wetlands. This might open new perspectives on the application of symbiotic AMF in the phytoremediation technology of wastewater in CWs.

Table 2.7. Studies investigating the effect of arbuscular mycorrhizal fungi on the purification of wastewater in constructed wetlands.

CWs type	Plant species	Study duration	Wastewater	Reference
VF-CWs	*Phragmites australis*	3 months	Textile bleaching wastewater	Hussain et al. (2019)
VF-CWs	*Brachiaria mutica*	27 days	Tannery wastewater	Ashraf et al. (2018)
VF-CWs	*Brachiaria mutica*	12 months	Industrial wastewater, textile production	Hussain et al. (2018a)
HF-CWs	*Scirpus validus*	24 hours	Domestic wastewater	Nimkar et al. (2012)
HF-CWs	*Brachiaria mutica*	24 hours	Domestic wastewater	Nimkar et al. (2012)
HF-CWs	*Phragmites australis*	3 months	Textile bleaching wastewater	Hussain et al. (2019)
HF-CWs	*Leptochloa fusca*	12 months	Industrial wastewater, textile production	Hussain et al. (2018b)
FT-CWs	*Brachiaria mutica*	8 days	Treated domestic wastewater mixed with raw industrial wastewater	Ijaz et al. (2015)
FT-CWs	*Brachiaria mutica*, *Leptochloa fusca*, *Phragmites australis*, and *Typha domingensis*	35 days	Domestic & industrial wastewater spiked with heavy metals	Shahid et al. (2020)

CWs type	Plant species	Study duration	Wastewater	Reference
FT-CWs	*Phragmites australis*	15 days	Drinking water spiked with phenol	Saleem et al. (2019)
FT-CWs	*Phragmites australis*	20 days	Textile bleaching wastewater	Nawaz et al. (2020)
FT-CWs	*Brachiaria mutica*, *Leptochloa fusca*, *Phragmites australis*, and *Typha domingensis*	42 days	Wastewater from crude oil production	Rehman et al. (2018, 2017)
FT-CWs	*Phragmites australis*	90 days	Diesel contaminated water	Fahid et al. (2020)
FT-CWs	*Phragmites australis*	24 months	Industrial wastewater, textile production	Shahid et al. (2020)
FT-CWs	*Canna indica* and *Cyperus alternifolius*	30	Domestic wastewater and salt	Gao et al. (2020)
VF-CWs	*Phragmites australis*	7 days	Synthetic polluted river water	Shao et al. (2014)
VF-CWs	*Phragmites australis*	7 days	Synthetic wastewater	Shao et al. (2014)

Note: FT-CWs: floating treatment constructed wetlands; VF-CWs: vertical flow constructed wetlands; HF-CWs: horizontal flow constructed wetlands.

2.3.2 Application of AMF in CWs

As mentioned above, AMF, which is widely present in various wetland habitats, has demonstrated the capacity to enhance the growth of wetland plants and promote plant tolerance to abiotic stresses. The application of AMF in wetland ecosystems has recently received greater research interest (**Table 2.7**). However, most of these studies were preliminary, which only focused on the effects of plant species or fungi species on the mycorrhizal status and the impacts of AMF on plant growth. Besides, some of them investigated the impact of AMF on plant tolerance to abiotic stresses in wetland systems (Huang et al., 2017a, 2017b, 2018; Wang et al., 2017a; Xu et al., 2019). For example, *Phragmites australis* inoculated with AMF significantly enhanced root growth under heavy metal stress compared to the control treatments. The enhancement was related to the added concentration of Cr (Wang et al., 2017a) or TiO_2 nanoparticles (Xu et al., 2019). However, studies on AM symbiosis have barely targeted the most relevant parameters for CW system operation, such as removal efficiency or treatment performance.

To date, only one study investigated the effect of AM symbiosis on the treatment efficiency in wetland systems (Gao et al., 2020). They found that floating treatment wetlands planted with inoculated *Phragmites* showed better removal performance of total dissolved solids, chemical oxygen demand, total phosphorus, and total nitrogen than non-inoculated control. Still, no statistical inferences were possible since the experimental design was not repeatedly treated. Recently, Palacios et al. (2021) investigated AMF inoculation in two plant species (*Ficinia nodosa* and *Carex appressa*) grown in stormwater biofilters, suggesting the positive effects of AMF on plant growth could directly improve nitrogen, phosphorus and Cr removal from stormwater, leading to a better performance of biofilters.

These indicated that the application of AMF seems a promising approach to improve the functioning of CWs for the purification of wastewater, but more studies are required to investigate further the potential roles of AMF in the removal of contaminants (e.g., N, P, heavy metals, and EOPs) in CWs.

2.3.3 Potential roles of AMF in EOPs removal in CWs

The symbiotic relationships between AMF and plant roots can be successfully established under the stress of EOPs (Wang et al., 2020). AMF could develop various strategies to persist in EOPs contaminated environment and avoid the damage produced by EOPs. Joner and Leyval (2003) found that the presence of AMF can enhance the growth of ryegrass (*Loliu mperenne* L.) and white clover (*Trifolium mrepens* L.) in soil with 2000 mg/kg of twelve different polycyclic aromatic hydrocarbons (PAHs). Experimental evidence has proven that AMF could promote the removal of PAHs (e.g., fluorene and phenanthrene) by enhancing plant (*Lolium multiflorum* Lam.) uptake and modifying the structure and density of bacterial populations in the mycorrhizosphere (Corgié et al., 2006; Gao et al., 2010). Similarly, Wu et al. (2008) also reported that AMF colonization (*Glomus etunicatum*) could enhance the uptake of dichlorodiphenyltrichloroethane by alfalfa roots, and significantly increased bacterial and fungal counts and dehydrogenase activity in the rhizosphere soil. Moreover, the positive effects of AMF on the biodegradation and phytoremediation of EOPs were also observed in the removal of benzo [*a*] pyrene (Liu et al., 2004), atrazine (Huang et al., 2007), PAHs (Yu et al., 2011), and petroleum contaminants (Małachowska-Jutsz and Kalka, 2010). In a recent review, Wang et al. (2020) suggested that AMF can alleviate the adverse effects of organic contaminant residues in crops through various mechanisms, such as promoting nutrient uptake and water acquisition, enhancing activities of contaminant degradation-related enzymes, alleviating oxidative stress of the host plant, and the accumulation and sequestration of pollutants by AMF structures.

In a pilot-scale CW system, Fester (2013) observed that the symbiotic relationships between AMF (*Funneliformis mosseae* and *Rhizophagus irregularis*) and plant roots (*Phragmites australis*) could rapidly and extensively establish under the stress of benzene, methyl *tert*-butyl ether and ammonia. This indicated that AMF might contribute to the removal of EOPs in CWs as a friendly and potentially biotechnological approach. However, there is no information about the application of AMF in CWs for the purification of wastewater containing EOPs. Further research is required to investigate the functional roles of AMF in EOPs removal in CWs and factors that affect the establishment of AM symbiosis (e.g., substrate types) to provide new insights into the application of AMF for EOPs removal in real-scale wetland systems.

Chapter III

Employ of arbuscular mycorrhizal fungi for pharmaceuticals ibuprofen and diclofenac removal in mesocosm-scale constructed wetlands

Bo Hu, Shanshan Hu, Zhongbing Chen, Jan Vymazal

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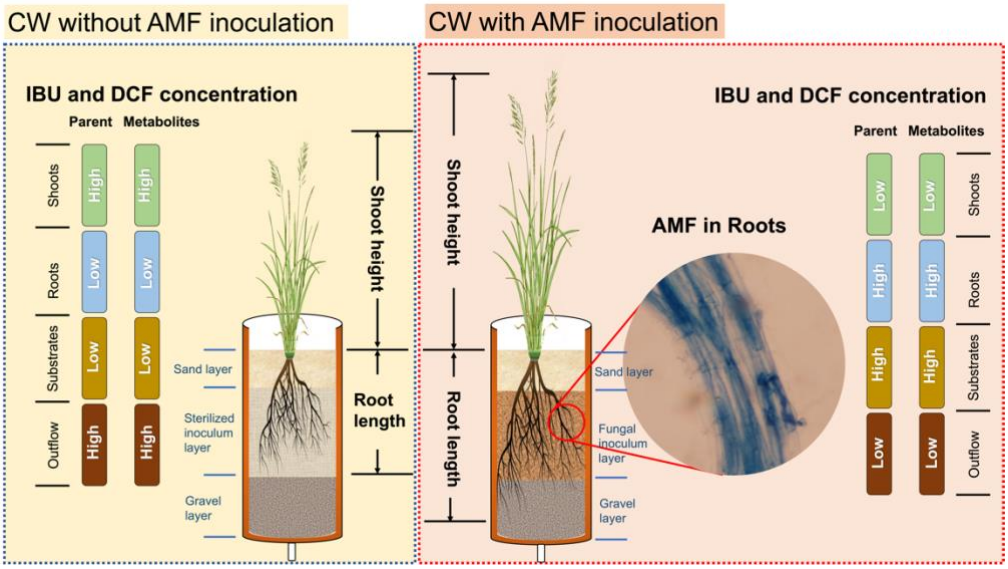
Contents

3.1 Abstract

This study investigated the effects of arbuscular mycorrhizal fungi (AMF) colonization on the growth of wetland plants (*Glyceria maxima*), and treatment performance in constructed wetlands (CWs) under the stress of pharmaceuticals ibuprofen (IBU) and diclofenac (DCF). Results showed that the growth of *G. maxima* was significantly increased by AMF colonization. AMF significantly increased the activities of antioxidant enzymes (peroxidase and superoxide dismutase) and soluble protein content in wetland plants, but the contents of malondialdehyde and $O_2^{\bullet-}$ were reduced. The removal efficiencies of TOC, PO_4^{3-}-P, NH_4^+-N, and TN were increased in AMF+ treatments by 6%, 11%, 15% and 11%, respectively. AMF increased the removal efficiencies of IBU and DCF by 6-14% and 2-21%, respectively, and reduced the content of their metabolites (2-OH IBU, CA IBU and 4'-OH DCF) in the effluent. Besides, the presence of AMF increased the contents of IBU and DCF in plant roots, while decreased their transportation to shoots. AMF symbiosis decreased the contents of IBU metabolites (2-OH IBU and CA IBU) but increased the contents of DCF metabolite (4'-OH DCF) in the roots of the host plant. In conclusion, these results indicated that AMF plays a promising role in CWs for emerging pollutants removal.

Graphical abstract:



CW: constructed wetland; AMF: arbuscular mycorrhizal fungi; IBU: ibuprofen; DCF: diclofenac.

3.2. Introduction

It has been reported that more than 1000 emerging organic pollutants (EOPs) which are currently not added into the traditional monitoring project by many countries have been detected in the environment according to the investigation of Norman Network (NORMAN Association, 2016). Many EOPs exhibit potential toxic effects on aquatic organisms due to their characteristics of bioaccumulation and difficult for biodegradation (Hao et al., 2007), which could eventually pose a risk to human health and ecosystem (Chen et al., 2017). Especially for some daily using pharmaceuticals with a huge consumption, such as ibuprofen (IBU) and diclofenac (DCF). They can be released continuously to the aquatic ecosystem, for example, non-negligible levels of IBU and DCF have been found in the range of 1.7~373.1 μg/L and 0.7~48.2 μg/L in the influent of wastewater treatment plant, respectively (Madikizela and Chimuka, 2017; Petrie et al., 2015; Santos et al., 2007). There are highly environmental concerns on IBU and DCF for aquatic life, including produce sex-specific responses (Flippin et al., 2007), genotoxic effects (Ragugnetti et al., 2011), influencing hatching, yolk sac, and tail deformation (Brandhof and Montforts, 2010). Moreover, conventional wastewater treatment plants have been proved to be one of the major sources to discharge EOPs into surface water (Farré et al., 2008). Therefore, the development of more effective wastewater treatment technology is an urgent need to prevent the release of EOPs into ecosystems.

Constructed wetlands (CWs) have been proved as a cost-effective wastewater treatment technology for the remove of EOPs (Vymazal et al., 2017), and have been reported as an alternative secondary wastewater treatment system to removal EOPs in many countries, such as Canada, Denmark, Italy, China, Singapore, the United States, and Spain (Li et al., 2014). The main removal mechanisms for EOPs in CWs including biodegradation, photodegradation, phytoremediation and sorption (Gorito et al., 2017). However, the negative effect of EOPs on the morphological and structural alterations of wetland plants was observed depending on EOPs' concentration and their duration of exposure (Bartha et al., 2014; Moro et al., 2014). Meanwhile, more and more studies reported a wide presence of various EOPs' metabolites in CW systems, and the metabolites may potentially cause greater risks than their parent EOPs (Han and Lee, 2017). The metabolites of IBU (such as hydroxy-IBU, 1,2-dihydroxy-IBU, carboxy-IBU, and glucopyranosyloxy-hydroxy-IBU) and DCF (such as hydroxy-DFC,

glucopyr-anosyloxy-hydroxy-DFC and DFC-glutathione) have been observed in the effluent, substrates, plant roots, and shoots in CWs (He et al., 2017; Matamoros et al., 2008; Bartha et al., 2014). Consequently, the discharge of various EOPs and their metabolites into the aquatic environment via CWs may bring new ecological concerns. Thus, it is necessary to improve the EOPs removal capacity in CWs to eliminate their potential negative impacts on the environments.

Arbuscular mycorrhizal fungi (AMF) as a common microorganism in the natural environment can form a mutualistic symbiosis with plants (James M Trappe, 1987). Numerous pieces of evidence pointed out that AMF can significantly improve the morphological, nutritional and physiological status of the host plants (Bao et al., 2019), thus promote the growth and stress tolerance of plants (Xu et al., 2018). For these reasons, AMF has become an important phytoremediation technology to restore contaminated soil or re-establish the degraded ecosystem such as abandoned agricultural fields and grassland (Richter and Stutz, 2002) and some abiotic stresses including drought, salinity, and heavy metals (Akbar Karimi, 2011; Hammer et al., 2015). In addition, AMF can protect plants from EOPs' damages by influencing the transport and distribution of EOPs in plant tissue. Previous studies reported that AMF contributed to reduce the concentration of EOPs in plant shoots by promoting the accumulation of EOPs in plant roots (Debiane et al., 2009; Langer et al., 2010). Due to the importance of AMF in protecting plants from adverse effects of EOPs and promoting associated microbes, the transformation and biodegradation of EOPs may be accelerated by AMF colonization in CWs. Recently, AMF has been shown to influence the biodegradation and phytoremediation of EOPs, such as improving the removal of polycyclic aromatic hydrocarbons and petroleum compounds in soil (Małachowska-Jutsz and Kalka, 2010; Yu et al., 2011). Fester (2013) reported that AMF (*Funneliformis mosseae* and *Rhizophagus irregularis*) can rapidly and extensively establish symbiosis in the roots of *Phragmites australis* under the stress of benzene, methyl *tert*-butyl ether and ammonia in a pilot-scale CW. This indicates that AMF may have an important function in wetlands, which might open new perspectives on the application of symbiotic AMF in the phytoremediation technology of wastewater contaminated with EOPs. However, there is no information so far about the effects of AMF on EOPs' removal in CWs.

Therefore, the aim of this study was to: 1) evaluate the effect of AMF on response strategies of wetland plant under the stress of IBU and DCF; 2) explore the role of AMF on the removal of IBU and DCF, as well as their metabolites in CWs. The results of

this study can provide an understanding of AMF's function in CW for phytoremediation applications.

3.3. Materials and methods

3.3.1 Chemicals and materials

Diclofenac sodium salt and ibuprofen of high purity grade (>98%) were purchased from Sigma-Aldrich. Their physicochemical capacities are given in **Table S3.1** in **Supporting Information (SI)**. *Glyceria maxima* were obtained from a local pond on the campus of the Czech University of Life Sciences Prague. Before cultivation, the roots of each seedling were washed carefully with tap water to remove soil and then sterilized in 75% ethanol for 10 seconds, followed by 1% NaClO solution for 10 minutes, finally washed five times with sterile distilled water. The fungal inoculum of AMF (*Rhizophagus irregularis*, BEG140) was obtained from the Institute of Botany, Czech Academy of Science, the composition of fungal inoculum was shown in **SI**.

3.3.2 Experimental setup

The CW systems were carried out in PVC-U materials columns with a dimension of 150×550 mm (diameter × height). Columns were divided into three layers, namely the bottom layer (0-150 mm), the middle layer (150-350 mm), and the top layer (350-550 mm) (**SI, Fig. S3.5**). The top and bottom layers in AMF- and AMF+ treatments contain the same substrates, filled with sand and gravel, respectively. The only difference in AMF- and AMF+ treatments is the different fungal inoculum added into the middle layer. For AMF+ treatments, 350 g fungal inoculum (*R. irregularis*, BEG140) was mixed with sand and then added into the middle layer of the column. For AMF- treatments, the fungal inoculum was replaced by 350 g of sterilized inoculum. Sand and gravel were sterilized at 120 ℃ for 3 h before filled into the column. Two seedings of *G. maxima* were transplanted into each column and set roots around the middle layer. Triplicates for each treatment and the details were shown in **SI, Fig. S3.5**. The experiment was carried out with rain protection in the natural environment. The temperature range during the experiment was -5~30 ℃ (**SI, Fig. S3.6**). The experiment

was ended when the temperature dropped below 0 ℃ (during the last week of the experiment).

CW systems fed with simulated municipal wastewater, and the details were shown in **SI, Table S3.2**. In order to maintain the growth of the plant and promote the formation of AMF symbiosis with plant roots, an intermittent hydraulic loading of 2 L/ 4 d (keep water for 2 h and then drain) was designed during the whole study period. Each column was irrigated with 10% strength synthetic wastewater in the first two months to avoid excessive nutrients. Since the beginning of the third month, the systems were run with 100% strength synthetic wastewater with the addition of ibuprofen (500 μg/L) and diclofenac (100 μg/L) until the end of the study. In total, the experiment operated continuously for five months from July to November.

3.3.4 Sample analysis

Conventional water quality parameters

Water samples were collected regularly from the influent and effluent of each CW every 8 days, and the effluent volume of each CW was recorded and shown in SI, Table S3. Total organic carbon (TOC) and total nitrogen (TN) were measured by the Primacs^{SERIES} TOC analyzer (Skalar, Dutch). PO_4^{3-}-P, NO_3^--N and NO_2^--N were analyzed by 883 Basic IC plus (Metrohm, Switzerland). NH_4^+-N was determined by the standard method (APHA, 2011).

Plant sampling and measurement

After two months of plant cultivation, and before the addition of IBU and DCF, AMF colonization in the roots of *G. maxima* was observed by using an optical microscope. Meanwhile, the mycorrhizal status in the plant's roots of each treatment was calculated. The detailed information of mycorrhizal assessment was shown in **SI**.

Plants were harvested at the end of the experiment, then divided into aboveground (stems and leaves) and belowground (roots and rhizomes). The root length and shoot

height were measured directly during the harvest with the fresh plants. Parts of fresh plant samples were prepared for the measurement of malondialdehyde (MDA), soluble protein, superoxide anion ($O_2^{\bullet-}$), superoxide dismutase (SOD) and peroxidase (POD), and the detailed analysis procedure was shown in **SI**. The rest plants were placed in a 40 ℃ oven for 120 h to prepare dry samples. Parts of dry plant samples were used for measuring the proportion of total carbon (TC) and TN in plants by the Primacs^{SN} analyzer (Skalar, Dutch). Parts of dry plant samples were powdered for EOPs measurement.

Analysis of pharmaceuticals and their metabolites

The content of IBU, DCF and their major metabolites, such as 2-hydroxy ibuprofen (2-OH IBU), carboxy ibuprofen (CA IBU) and 4'-hydroxy diclofenac (4'OH DCF) in water (influent and effluent), rhizosphere soil, dried plant roots and shoots were analyzed by liquid chromatography-mass spectrometry (LC-MS). The detailed analysis procedure was shown in **SI**.

3.3.5 Data analysis

Mass removal efficiencies of pollutants, including TOC, TN, P, NH_4^+-N, IBU and DCF, were calculated based on the effective mass balance of pollutants in the influent and effluent by the equation given below.

$$\text{Mass removal efficiency (\%)} = \frac{V_{in} \times C_{in} - V_{out} \times C_{out}}{V_{in} \times C_{in}} \times 100 \qquad (1)$$

Where V_{in} and V_{out} are the volumes of the influent and effluent, C_{in} and C_{out} are the concentration of pollutants from influent and effluent.

In order to investigate the effect of AMF on the metabolic pathway (aerobic and anaerobic degradation) of EOPs, we used the equations (Eq. (2) and Eq. (3)) introduced by Matamoros et al. (2008) to calculate the removal percentage of IBU by aerobic and anaerobic pathways. These equations were based on the differences in IBU metabolites (2-OH IBU and CA IBU) under aerobic and anaerobic conditions.

$$\text{Aerobic degradation ratio} = \frac{C_{2-OH\ IBU} + x * C_{CA\ IBU}}{C_{2-OH\ IBU} + C_{CA\ IBU}}\ x\ 100 \quad (2)$$

$$\text{Anaerobic degradation ratio} = \frac{y * C_{CA\ IBU}}{C_{2-OH\ IBU} + C_{CA\ IBU}}\ x\ 100 \quad (3)$$

Where C_α is the concentration of IBU metabolite α, The values of x and y are 0.4% and 1.8%, respectively, indicating the percentage of CA-IBU obtained in the aerobic and anaerobic pathways (Zwiener et al., 2002).

Data are presented as mean and standard errors from data of the tree parallel treatments. Plant biomass, nutrients in plant tissues, MDA, $O_2^{\bullet-}$, antioxidant enzymes, soluble protein, the content of IBU and DCF and their metabolites were statistically evaluated using SPSS (version 28.0) software package (Chicago, IL, USA). The student's t-test was used to compare treatment differences with $p < 0.05$ set as a significant difference. Additionally, principal compounds analysis (PCA), Spearman's correlation analysis and cluster analysis were applied to examine the impacts of AMF on wetland plant growth under the stress of IBU and DCF. 'Factoextra' (Kassambara, 2017), 'Corrplot' (Wei and Simko, 2019) and 'Pheatmap' (Kolde and Kolde, 2015) packages were used by R Software (version 3.6.3) to visualize the experimental data among plant growth, physiological indexes in plant roots, IBU, DCF and their metabolites in AMF- and AMF+ treatments.

3.4 Results and discussion

3.4.1 AMF colonization in wetland plant

AMF colonization in the roots of *G. maxima* was observed in the AMF+ treatments (**SI, Fig. S3.1**). The frequency of mycorrhiza (F%) is 61.34%, and the intensity of mycorrhizal colonization (M%) is 11.24% (**Table 3.1**). This result is in good agreement with Ray and Inouye (Ray and Inouye, 2006) who studied the effect of intermittent flows on AMF colonization and suggested that the length of the unflooded period shows a positive correlation with the hyphal and arbuscular colonization of *T. latifolia*. This was ascribed to the oxygenation of the rhizosphere during the exchange of wet and dry periods (Liang et al., 2018a), which provides suitable oxygen for the development of AMF colonization in CWs. Meanwhile, plants

can be colonized by AMF because of the aerenchyma structure in wetland plants that can provide active ventilation of the roots and rhizomes, and thus maintain favorable oxygen conditions for AMF growth (Dickopp et al., 2011). However, due to the limit of oxygen in wetland ecosystems, the frequently anoxic conditions always conducted negative effects on the processes of fungal root colonization. As a result, AMF colonization in most wetland plant roots was still at a low level (< 25%) (Wang et al., 2018a). Hu et al. (2020b) investigated AMF colonization in two wetland plants (*S. sylvaticus* and *P. arundinacea*) under different water regimes and found that AMF colonization in low water regime was more frequent in comparison with high water regime. Similar results were also reported by Wolfe et al. (2007) who found that the decrease of AMF colonization in the high water regime mainly due to the lacked available oxygen for AMF growth in wetlands. Therefore, the symbiosis of AMF and wetland plants can be established in wetlands by using fluctuating water or intermittent regime.

Table 3.1. Mycorrhizal status under different treatments. These include the frequency of mycorrhiza in the root system (F%), the intensity of mycorrhizal colonization (M%) and arbuscule abundance (A%) in the whole root system. Data are presented as means ± SD.

Treatments	AMF colonization		
	F%	M%	A%
AMF-	0	0	0
AMF+	61.3±10.48	11.2±2.47	0.5±0.07

3.4.2 Effect of AMF on plant growth and physiological indexes

Biomass and nutrients in wetland plants

The shoot height, root length, shoot weight and root weight of *G. maxima* in the AMF+ treatment was significantly ($p < 0.05$) higher than in the AMF- treatment, with an increase of 19.78%, 23.62%, 53.27%, and 21.50%, respectively (**Table 3.2**). There

are significant increases ($p < 0.05$) in the TN and TC content in plant roots in the CWs with AMF inoculation. The TN content is 1.50% in the AMF- treatments, and 2.04% in the AMF+ treatments. The TC content is 24.90% in the AMF- treatments, and 31.45% in the AMF+ treatments. Therefore, AMF had a positive effect on the growth of wetland plants even under the stress of EOPs.

Table 3.2. The effects of AMF on the growth and nutrients of *G. maxima*. a and b show the significant difference between AMF- and AMF+ treatment ($p < 0.05$). TN: total nitrogen; TC: total carbon.

Plant tissues	Parameters	Units	Treatments	
			AMF-	AMF+
Roots	Length	cm	18.33±2.08[a]	22.66±1.15[b]
	Dry Weight	g	12.16±1.08[a]	15.99±1.07[b]
	TN	%	1.50±0.18 [a]	2.04±0.11 [b]
	TC	%	24.90±2.32 [a]	31.45±3.16[b]
Shoots	Length	cm	33.67±2.08[a]	40.33±1.53[b]
	Dry Weight	g	9.18±1.21[a]	14.07±0.33[b]
	TN	%	3.78±0.22 [a]	3.27±0.69 [a]
	TC	%	44.70±1.23 [a]	42.04±2.84 [a]

In general, AMF symbiosis contributes to the beneficial effect on plant growth under abiotic stress. Prosser et al. (2014) found that AMF symbiosis improves the growth of radish, carrot, soybean, lettuce, and wheat plants under the stress of triclosan and triclocarban. A possible explanation of this advantage might be that AMF can affect the physiological and biochemical response of plants to reduce the negative impacts of adverse stress. Moreover, a positive effect on *G. maxima* growth may also be related to the improvement of nutrients and water accomplished by the dense extra-radical mycelium network, which extends the scope of plant roots to obtain nutrients in intermittent loading CWs (Jansa et al., 2019). Additionally, previous studies demonstrated that AMF can deliver up to 80% P and 42% N into the plant to promote host plant growth (Marschner and Dell, 1994). Hu et al. (2020b) showed that TP, TC,

TN contents and biomass of inoculated wetland plants (*S. sylvaticus* and *P. arundinacea*) were significantly higher ($p < 0.05$) than that of the corresponding non-inoculated plants under fluctuating water regime. Therefore, AMF had a positive effect on wetland plants, including the growth, biomass, and nutrients uptake, even under EOPs' stress.

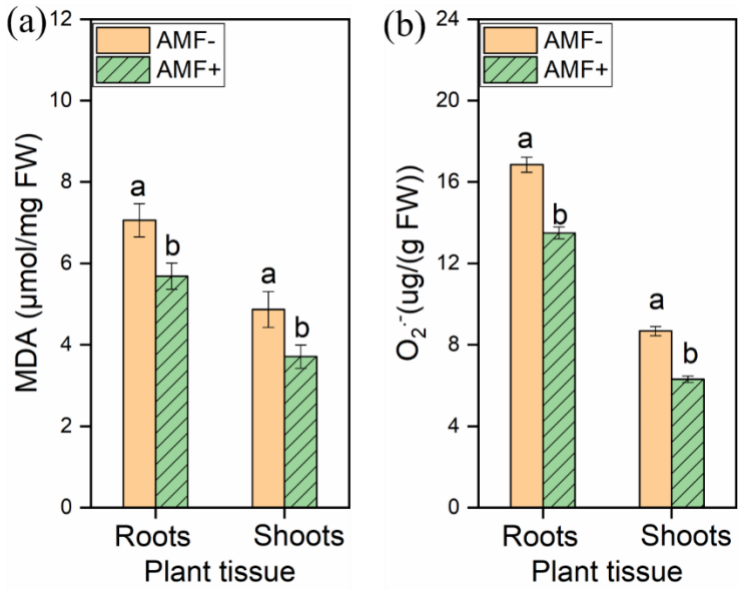


Fig. 3.1. (a) MDA content in plant tissues. (b) $O_2^{\bullet-}$ content in plant tissues. The data are the means ± standard errors (n=3). a and b show the significant difference in AMF- and AMF+ treatments ($p < 0.05$). MDA: malondialdehyde; $O_2^{\bullet-}$: superoxide anion.

ROS levels and lipid peroxidation under EOPs stress

Superoxide anion ($O_2^{\bullet-}$) is one of the main reactive oxygen species (ROS) produced in plants under a variety of abiotic stresses, which might cause biomolecule damages and lead to severe lipid peroxidation and oxidative stress (Asgari Lajayer et al., 2017; Jung, 2004). MDA has been considered as an indicator of lipid peroxidation produced in plants under the oxidative stresses (Aibibu et al., 2010). Hence, lipid peroxidation is closely relevant to the ROS level. Compared with AMF- treatments, the MDA content in both roots and shoots of *G. maxima* in AMF+ treatments were

decreased by 24.83% and 21.97%, respectively, indicating that AMF decreased lipid peroxidation of the inoculated plant under EOPs stress (**Fig. 3.1**). Moreover, the $O_2^{\bullet-}$ generation rate in both roots and shoots of the inoculated plant was lower than that of non-inoculated plants, with a decrease of 25.33% and 18.94%, respectively.

AMF can decrease the production of ROS and MDA under a variety of abiotic stresses and it contributes to ameliorate the effect of oxidative stress to host plants (Janeeshma and Puthur, 2020). In this study, ROS level and lipid peroxidation significantly decreased ($p < 0.05$) in *G. maxima* with AMF inoculation, indicating that AMF could alleviate or decrease oxidative injuries of plants conducted by the stress of IBU and DCF. In the other two wetland plants *S. sylvaticus* and *P. arundinacea*, Hu et al. (2020b) reported that AMF significantly decreased ($p < 0.05$) the MDA content in both plants under drought stress. Xu et al. (2019) studied the effects of *F. mosseae* on *P. australis* (reed) under different TiO_2 nanoparticles concentrations and found that *F. mosseae* significantly decreased the contents of MDA and ROS level ($p < 0.05$). The reduction of MDA and ROS were 38-67% and 2-49%, respectively. Therefore, AMF might be beneficial to alleviate oxidative injuries of wetland plants under the stress of IBU and DCF.

Antioxidant response under EOPs' stress

SOD, POD and soluble protein in *G. maixma* tissues were significantly higher in the AMF+ treatments than that in the AMF- treatments under the stress of IBU and DCF (**Fig. 3.2**). SOD, POD and soluble protein content in the *G.maxima* roots of inoculated CWs were significantly higher than in the non-inoculated CWs ($p <0.05$), with an increase of 26.85%, 32.06% and 21.49%, respectively. Meanwhile, the contents of SOD, POD and soluble protein in shoots were also significantly higher than that of non-inoculated *G. maxima*, with an increase of 18.67%, 18.09% and 27.98% respectively. These results indicated that AMF had a positive effect on promoting the antioxidant enzyme activities and soluble protein of wetland plants under the stress of IBU and DCF.

POD and SOD both are the antioxidant enzymes, which can trigger an antioxidant response to convert ROS into non-toxic molecules (Wang et al., 2018c). Ferrol (2016) reported that AMF can enhance plant stress tolerance by increasing the expression of genes related to the synthesis of antioxidant enzymes, such as POD and SOD (Ren et

al., 2019). Similar to our study, Wu et al. (2020) reported that AMF can relieve the photosynthesis inhibition of *P. australis* conducted by Cu stress through up-regulating the expression of ferredoxin-NADP+ reductase (FNR, relating to photosynthesis electron transport) and CP43 (Photosystem II light-harvesting protein). Moreover, AMF promoted the secretion of glycoproteins into the rhizosphere to make host plants more efficient in nutrient uptake and enhance plant tolerance to abiotic stress (Aranda et al., 2009). Therefore, AMF appears to protect *G. maxima* from the stress of IBU and DCF and promote plant growth by hormonal regulation, such as increasing antioxidant enzyme activities (SOD and POD) and soluble protein contents.

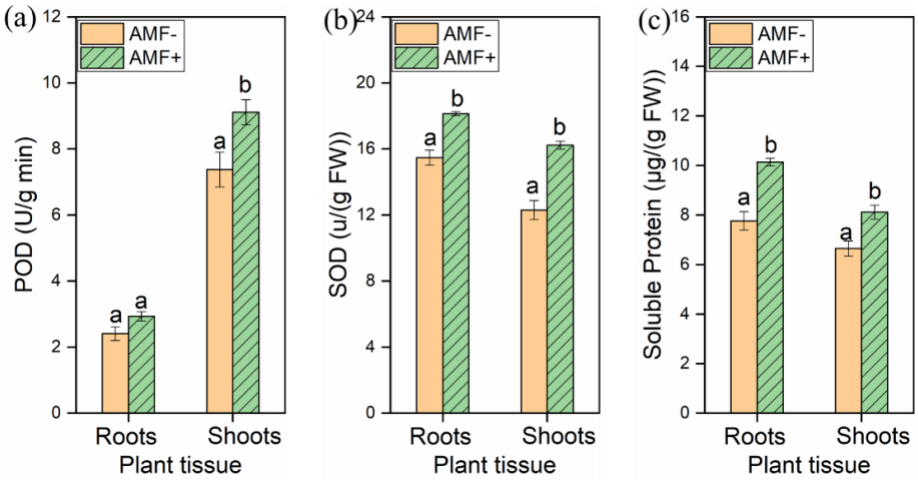


Fig. 3.2. Antioxidant enzyme activities and soluble protein contents in plant tissues: (a) POD, (b) SOD, and (c) Soluble protein. The data are the means ± standard errors (n=3). a and b show the significant difference in AMF- and AMF+ treatment ($p < 0.05$). POD: peroxidase; SOD: superoxide dismutase.

3.4.3 Effect of AMF on conventional water quality

The average TOC mass removal efficiency for AMF- and AMF+ treatments were 85.44% and 91.63%, respectively (**SI, Fig. S3.2**). The average PO_4^{3-}-P mass removal efficiency in AMF+ treatments was 10.92% higher than in the AMF- treatments (**SI, Fig. S3.3**). The TN mass removal efficiency in AMF+ treatments was 7.47% higher than that in the AMF- treatments (**SI, Fig. S3.4**). For NH_4^+-N, the average mass removal

efficiency in AMF- and AM+ treatments were 72.42% and 87.24%, respectively. The NO_2^--N was not added in the synthetic wastewater (**SI, Table S3.1**), however, the NO_2^--N was quickly generated in all treatments as the intermediate products of nitrification, with the mean concentration of 1.08 mg/L and 1.39 mg/L in AMF- and AMF+ treatment effluents, respectively. Additionally, the NO_3^--N mean concentration in the effluents of AMF- and AMF+ treatment was 20.84 mg/L and 22.88 mg/L, respectively, which was higher than that in the influents (5.14 mg/L).

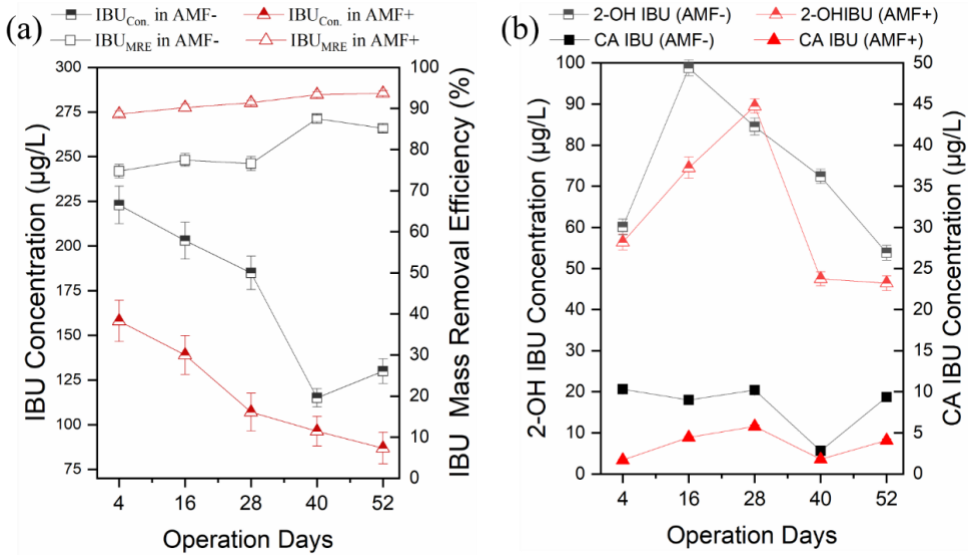


Fig. 3.3. (a) Ibuprofen concentration ($IBU_{Con.}$) and its mass removal efficiency (IBU_{MRE}) in AMF- and AMF+ treatments after the addition of EOPs. (b) The concentration of ibuprofen's metabolites, 2-hydroxy ibuprofen (2-OH IBU) and carboxy ibuprofen (CA IBU), in the liquid phase after the addition of EOPs. The data are the means ± standard errors (n=3).

AMF can increase organic matter removal performance in CWs. It is mainly due to that AMF improves plant growth via promoting plant uptake of nutrients such as P, N, and some micronutrients (Wang et al., 2017b). There are two ways to transport nutrients in inoculated plants: direct (plant) uptake pathway and mycorrhizal uptake pathway, while the non-inoculated plant gets nutrients only by direct pathway (Bücking and Kafle, 2015). However, the mycorrhizal pathway is more efficient compared with the direct pathway. AMF developed extensive and highly branched external mycelium to absorb nutrients far beyond the area of rhizosphere and transferred to the intraradical mycelium, providing nutrients for host plants, in return, host plant supply carbon source

(in the form of sucrose) to maintain the growth of AMF (Schaarschmidt et al., 2006). Hu et al. (2020b) proved that the TP and TN contents of wetland plants (*P. arundinacea* and *S. sylvaticus*) were significantly increased by AMF colonization. Additionally, the high organic matter removal efficiency of AMF+ treatments in our study was related to the well-development roots of *G. maxima*, which provided more oxygen for microbial metabolism, and thus enhancing aerobic microbial processes, such as TOC removal and nitrification (Stein and Hook, 2005). Therefore, AMF exhibits an important function in promoting organic matter removal efficiency in CWs.

3.4.4 The fate of IBU and DCF in AMF assistant CWs

IBU and DCF in the liquid phase

The removal efficiencies of IBU in AMF- and AMF+ treatments were 74.78-87.56% and 88.66-93.71%, respectively (**Fig. 3.3a**). Meanwhile, the removal efficiencies of DCF in AMF- and AMF+ treatments were 53.75-66.08% and 70.98-79.68%, respectively (**Fig. 3.4**). The removal efficiency of IBU and DCF was significantly affected by the addition of AMF ($p < 0.05$). The mass removal efficiencies of IBU and DCF in AMF+ treatments were 5.82-13.88% and 2.32-21.07% higher than those in AMF- treatments. These suggested that AMF had a positive effect on IBU and DCF removal in CWs. Furthermore, lower concentrations for the metabolites of IBU and DCF were detected in the AMF+ CWs than in the AMF- CWs (**Fig. 3.3b and Fig. 3.4**). The concentrations of 2-OH IBU, CA IBU and 4'-OH DCF in AMF- treatments were 53.80-98.80 μg/L, 2.80-10.30 μg/L and 0.04-0.72 μg/L, respectively; while those in AMF+ treatments were 46.40-89.50 μg/L, 1.70-5.78 μg/L and 0.11-0.27 μg/L, respectively. These indicated that AMF could promote the degradation of IBU and DCF as well as their metabolites in CW systems.

AMF showed positive effects on decreasing the effluent concentration of IBU and DCF and their metabolites in CWs. The possible reason was that AMF promoted nutrient uptake of the host plant and reduced the adverse effects of pharmaceuticals' stress, thereby improving plant growth (**Table 3.2 and Fig. 3.1, 3.2**). Then the significantly higher root length of the inoculated plants might enhance the oxygen exchange between plant roots and the rhizosphere, creating an aerobic condition that

are more conducive to the degradation of IBU and DCF. It has been reported that the aerobic condition has a high correlation with IBU and DCF removal in CW systems (Nivala et al., 2019). Similarly, Zhang et al. (2012) and Ávila et al. (2013) also proved that the dissolved oxygen in the substrate pores in CWs can play an important role in promoting the removal of pharmaceuticals, such as IBU, DCF and ketoprofen. Under the aerobic conditions, IBU produced 2-OH IBU and CA IBU through the conjugation with glucuronic acid and oxidation (Zwiener et al., 2002), while DFC produced 4'-OH DCF through hydroxylation reaction as the initial step of transformation (Bouju et al., 2016). AMF showed a positive effect on enhancing the aerobic degradation of EOPs by analyzing the metabolic pathway of IBU. The aerobic degradation of IBU in AMF+ treatments was 5.17% higher than that in AMF- treatments (**Table 3.3**). Moreover, the positive effect of aerobic conditions on the removal of IBU metabolites also was reported by the previous studies (Matamoros et al., 2009). They found that more than 99% of 2-OH IBU and CA IBU were removed under the aerobic conditions in unsaturated vertical flow CW, while the removal rate of 2-OH IBU and CA IBU under anoxic conditions in saturated vertical flow CW were 50% and 71%, respectively. Furthermore, the effect of AMF colonization on the transport and distribution of IBU and DCF in the host plant may be another reason for the better removal efficiency of IBU and DCF in AMF+ treatments. Huang et al. (2007) reported that AMF enhanced the accumulation of atrazine in the roots of maize and the atrazine concentration decreased markedly in the rhizosphere and near-rhizosphere of AMF treatment. Similarly, Wu et al. (2009) reported that the presence of AMF enhances the accumulation of phenanthrene in *Medicago sativa* L.

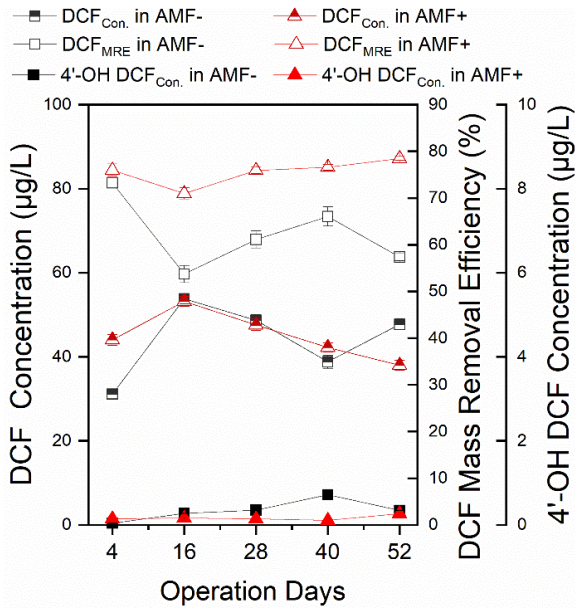


Fig. 3.4. Concentration of diclofenac ($DCF_{Con.}$) and its metabolites (4'-hydroxy diclofenac, 4'-OH $DCF_{Con.}$) in the liquid phase, and the mass removal efficiency of diclofenac (DCF_{MRE}) in AMF- and AMF+ treatments after the addition of EOPs. The data are the means ± standard errors (n=3).

Table 3.3. Ibuprofen metabolic pathway in AMF- and AMF+ treatment systems.

Date	AMF- Treatments		AMF+ Treatments	
	aerobic degradation ratio (%)	anaerobic degradation ratio (%)	aerobic degradation ratio (%)	anaerobic degradation ratio (%)
9/12	85.43	0.26	97.09	0.05
9/24	91.68	0.15	94.37	0.10
10/6	89.27	0.19	93.96	0.11
10/18	96.29	0.07	96.38	0.07
11/3	85.23	0.27	91.93	0.15
Mean	89.58	0.19	94.75	0.09

Additionally, the difference in removal efficiency between IBU and DCF in CWs is expected to strongly depend on their physicochemical properties (Onesios et al., 2009). The functional groups and small changes in chemical structure may have significant effects on solubility and polarity of EOPs, as well as their biodegradability (Imfeld et al., 2009). The low removal efficiency of DCF might be related to the presence of chlorine in its structure (Zorita et al., 2009). According to Ghattas et al. (2017), the further transformation of DCF, which is dechlorination, occurs under anaerobic conditions. The dechlorination reaction of DCF in this studied CW system was probably inhibited by the lack of reducing conditions. Similar to our results, Matamoros and Bayona (2006) investigated the removal efficiency of 11 EOPs in two CWs and found that the removal rate of DCF was no greater than 45%, while an 80% removal rate of IBU was observed.

IBU and DCF in plant tissues and rhizosphere soil

AMF had significant effects on the uptake of IBU and DCF by plant roots ($p < 0.05$) (**Table 3.4**). The content of IBU and DCF in plant roots were increased by 33.14% and 143.11% in the AMF+ CWs compared to the AMF- CWs. On the contrary, both IBU and DCF contents in plant shoots of AMF- CW were 10.0 μg/kg and 10.5 μg/kg, respectively, but they were not detected (below the detection limit) in plant shoots of AMF+ CWs. This indicates that AMF promotes the uptake of parent EOPs (IBU and DCF) in the roots of wetland plants and restricts their transportation from roots to shoots. Moreover, the contents of 2-OH IBU and CA IBU (metabolites of IBU) in plant roots of AMF+ CWs were significantly lower than those in AMF- CWs ($p < 0.05$). However, the content of 4'-OH DCF (a metabolite of DCF) in the plant roots of AMF+ CWs was significantly higher than that in the AMF- CWs ($p < 0.05$). Besides, IBU, 2-OH IBU, CA IBU and DCF were not detected in the rhizosphere soil in the AMF- CWs (below the detection limit), while they were observed in rhizosphere soil of AMF+ CWs with the content of 111.0 μg/kg, 45.1 μg/kg, 20.2 μg/kg and 33.4 μg/kg, respectively. This indicates that AMF may also have positive effects on promoting the accumulation of IBU and DCF in the substrate of CWs.

Table 3.4. The contents of ibuprofen, diclofenac and their metabolites in the rhizosphere soil and *G. maxima* tissues (roots and shoots). a and b show the significant difference for AMF- and AMF+ treatment ($p < 0.05$). n.d.: not detected; IBU: ibuprofen; DCF: diclofenac; 2-OH IBU: 2-hydroxy ibuprofen; CA IBU: carboxy ibuprofen; 4'-OH DCF: 4'-hydroxy diclofenac.

Compounds	Treatments	Rhizospheres soil (µg/kg)	Roots (µg/kg)	Shoots (µg/kg)
IBU	AMF-	n.d.	69.7±1.8 [a]	10.0±0.4
	AMF+	111.0±6.6	92.8±1.5 [b]	n.d.
2-OH IBU	AMF-	n.d.	155±3.6 [b]	85.2±2.4 [b]
	AMF+	45.1±1.2	104.0±3.4 [a]	73.4±2.2 [a]
CA IBU	AMF-	n.d.	27.9±0.4 [b]	n.d.
	AMF+	20.2±0.5	18.1±0.5 [a]	n.d.
DCF	AMF-	n.d.	61.7±2.5 [a]	10.5±0.3
	AMF+	33.4±1.2	150±3.8 [b]	n.d.
4'-OH DCF	AMF-	n.d.	65.2±4.0 [a]	n.d.
	AMF+	n.d.	163.0±2.8 [b]	n.d.

AMF enhanced the uptake of IBU and DCF in the roots of *G. maxima* but inhibited their transportation from root to shoot. The possible reason is that AMF colonization attributed to the increased EOPs adsorption on roots by specific mycelium structures. Plant uptake is considered one of the main removal mechanisms of EOPs in CW systems (Matamoros and Bayona, 2008), however, plant uptake and translocation of EOPs into plant roots from solution were directly proportional to log K_{ow} (or inversely proportional to water solubility) (Topp et al., 1986). This is an explanation that similar contents of DCF and IBU were observed in the plant tissue (both in roots and shoots) from non-inoculated CWs, because of their similar log K_{ow} value (3.26 of IBU and 4.26 of DCF) (**SI, Table S3.1**), despite the added concentration of DCF was 5 times lower than that of IBU.

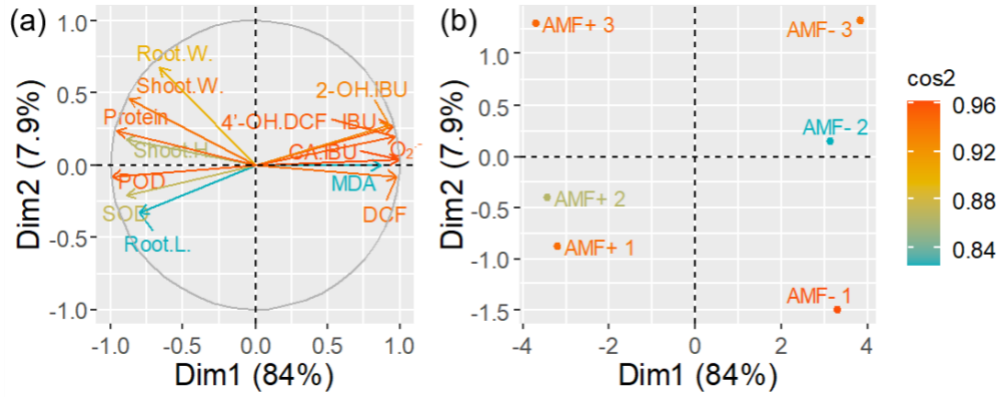


Fig. 5. (a) Principal component analysis (PCA) among plant growth, physiological indexes in plant roots, IBU and DCF as well as their metabolites in AMF- and AMF+ treatments. (b) The scores of each treatment on Dim1 and Dim2. AMF- 1, 2 and 3 represent the triplicates of AMF- treatments. AMF+ 1, 2 and 3 represent the triplicates of AMF+ treatments. IBU: ibuprofen; DCF: diclofenac; 2-OH IBU: 2-hydroxy ibuprofen; CA IBU: carboxy ibuprofen; 4'-OH DCF: 4'-hydroxy diclofenac; Root W.: Root weight; Root L.: Root length; Shoot W.: Shoot weight; Shoot H.: Shoot height.

The mycorrhizal pathway may be another non-negligible approach to translocate EOPs between plant tissues and CW systems. Although the existing results of our study still lacking evidence for the direct involvement of AMF in EOPs removal, Gao et al. (2010) used three-compartment systems to investigate the effects of AMF on EOPs uptake by plant roots and proved that the mycorrhizal pathway contributes to the uptake of polycyclic aromatic hydrocarbons by plant roots. Gao et al. (2010) found that the extraradical hyphae of AMF can extend into the EOPs-spiked compartment, then absorb and transported EOPs to the roots of inoculated plants grown in an un-spiked compartment, resulting in high contents of fluorene and phenanthrene in the roots of inoculated ryegrass (*Lolium multiflorum* Lam.). Similar to our results, Huang et al. (2007) reported that AMF significantly increased atrazine concentration in the roots of maize, while greatly decreased its concentration in shoots. Wu et al. (2009) testified the presence of AMF promotes the accumulation of phenanthrene in epidermal cells of roots and reduced the transport into the root interior and shoots. Therefore, IBU and DCF might also be absorbed by the external mycelium developed by AMF and transported to arbuscular structures inside the roots, resulting in notable contents of IBU and DCF in the roots of inoculated *G. maxima*. However, whether the mycorrhizal pathway is directly involved in the translocation and transformation of EOPs by wetland plants needs to be studied further. For example, a study on compartmented

cultivation systems with a separate hyphal compartment or supplying additional nutrients for AMF- treatment to match the biomass of inoculated plants.

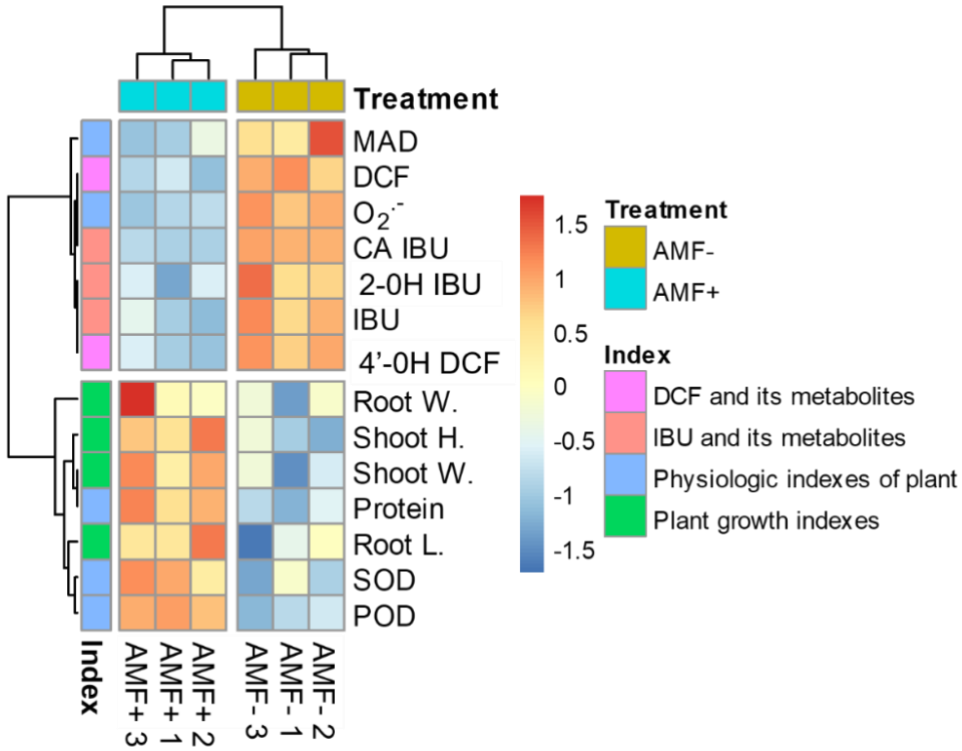


Fig. 3.6. The cluster analysis among plant growth, physiological indexes (in plant roots, IBU and DCF as well as their metabolites in AMF- and AMF+ treatments. IBU: ibuprofen; DCF: diclofenac; 2-OH IBU: 2-hydroxy ibuprofen; CA IBU: carboxy ibuprofen; 4'-OH DCF: 4'-hydroxy diclofenac; Root W.: Root weight; Root L.: Root length; Shoot W.: Shoot weight; Shoot H.: Shoot height. Colors in the heatmap indicate the correlation between the different data sets.

Furthermore, the content of IBU metabolites, 2-OH IBU and CA IBU, in inoculated plant's tissue was higher, compared with non-inoculated plant's tissue. On the contrary, the contents of 4'-OH DCF in inoculated plant's roots were 2.5 times higher than that of non-inoculated plant's roots. The possible reason might be attributed to AMF promoting gene expression related to the metabolism of IBU and DCF. Although there is no relevant data on AMF affecting gene expression in this study, some studies have confirmed that AMF can significantly increase the activities of

enzymes, such as cytochrome P450 monooxygenase (P450) (Bellés et al., 2008), glycosyltransferase (GT) (Chen et al., 2018), glutathione-S transferase (GST) (García-Sánchez et al., 2014). Among such enzymes, He et al. (2017) and Bartha et al. (2014) confirmed that the activities of P450, GT and GST have a positive correlation with the metabolism of IBU and DCF in *P. australis* and *Typha latifolia*. Therefore, the lower concentration of 2-OH IBU and CA IBU in inoculated plant roots was mainly due to the further biodegradation of IBU into other metabolites, such as 1-hydroxy-IBP, 1,2-dihydroxy-IBP and glucopyranosyloxy-hydroxy- IBP, which have been reported by He et al. (2017). Although oxygenation of the chlorinated benzene ring enables further biodegradation of 4'-OH DCF, this process is much more likely under anaerobic conditions (Ghattas et al., 2017). The further transformation of 4'-OH DCF was probably inhibited by the lack of reduction conditions, resulting in the excess content of 4'-OH DCF accumulated in inoculation plant roots. Additionally, it has been proved that AMF colonization can increase the amount and quality of host root exudates (Scheffknecht et al., 2006), including some lipophilic compounds, flavonoids, amino acids, protein, and other biomolecules, which could contribute to the accumulation of IBU and DCF and their metabolites in the rhizosphere soil.

3.4.5 Effect of AMF on plant detoxification

In order to investigate the potential functional role of AMF on plant detoxification of IBU and DCF, PCA and cluster analysis were conducted to analyze the data including plant growth indexes, physiological indexes of plant roots and IBU, DCF as well as their metabolites in plant roots. PCA results showed that the first two components Dim1 and Dim2 account for 84% and 7.9% variability, respectively, indicating that those parameters were largely determined by Dim1 (**Fig. 3.5**). On Dim1, the root length, root weight, shoot height, shoot weight, protein, SOP and SOD clearly showed that plant growth indexes, antioxidant enzymes and soluble protein were highly dependent upon each other (r is 0.75 to 1) under the stress of IBU and DCF, while the concentrations of IBU, DCF and their metabolites (IBU, DCF, 2-OH IBU, CA IBU, and 4'-OH DCF) in plant roots had a strong negative correlation (r is -0.75 to -0.99) with oxidative damage (MDA and $O_2^{\bullet-}$). Meanwhile, according to the scores of each system on Dim1 and Dim2, significant statistical differences can be observed from the two distinct groups: AMF- and AMF+ treatments. Similarly, the results of hierarchical clustering analysis also indicated that plant growth indexes, POD, SOD, and protein were highly positively correlated with AMF+ treatment but were negatively correlated

with AMF- treatments (**Fig. 3.6**). IBU, DCF, and their metabolites showed a positive correlation with oxidative damage of *G. maxima* in AMF- treatments but a negative correlation in AMF+ treatments. Thus, AMF colonization showed positive effects on wetland plants detoxification of IBU and DCF by enhancing hormone regulation, such as increasing the release of antioxidant enzymes and soluble protein to decrease oxidative damage and promote plant growth.

3.5 Conclusion

AMF symbiosis with wetland plants has an important function in CWs. It can significantly promote the growth of *G. maxima* and nutrients uptake under the stress of IBU and DCF by increasing antioxidant enzymes (POD and SOD) and soluble protein contents to decrease oxidative damage ($O_2^{\bullet-}$ and MDA). AMF can improve the treatment performance of conventional pollutants in CWs with the removal efficiency of TOC, PO_4^{3-}-P, NH_4^+-N, TN in AMF+ treatments significantly improved by 6.19%, 10.92%, 14.82% and 10.92%, respectively. Meanwhile, AMF colonization could be an effective strategy to enhance IBU and DCF removal in CW systems, in which the removal efficiencies of IBU and DCF can increase 5.82-13.88% and 2.23-21.07%, respectively. At the same time, it can reduce the concentrations of their metabolites (2-OH IBU, CA IBU and 4'-OH DCF) in the effluent. Moreover, AMF may relocate the transformation of IBU and DCF in plant tissues with higher accumulation in the roots and lower transportation to the shoots. Besides, the presence of AMF also contributed to the accumulation of IBU and DCF and their metabolites in rhizosphere soils. Overall, this study provides encouraging evidence that the introduction of AMF into CWs can enhance the removal of IBU and DCF as well as conventional pollutants. However, more in-depth research is needed on how AMF helps wetland plants to remove IBU and DCF.

3.6 Supporting information

Methods and materials

- The composition of fungal inoculum and sterilized inoculum
- Mycorrhizal assessment
- Determination of MDA, $O_2^{\bullet-}$, POD, SOD, and soluble protein
- Determination of EOPs and their metabolites

Tables:

- Table S3.1. Physical-chemical capacities of EOPs and their metabolites.
- Table S3.2. The characteristics of simulated sewage
- Table S3.3. Mean volume of each system effluent

Figures:

- Fig. S3.1. AMF colonization in the roots of *G. maxima* in AMF- and AMF+ treatments.
- Fig. S3.2. TOC concentration and its mass removal efficiency in AMF- and AMF+ treatments.
- Fig. S3.3. Phosphorus (P) concentration and its mass removal efficiency in AMF- and AMF+ treatments.
- Fig. S3.4. TN, NH_4^+-N, NO_3^--N and NO_2^--N in AMF- and AMF+ treatments.
- Fig. S3.5. Sketch of CWs reactors
- Fig. S3.6. The temperature changes during the experiment

Methods and materials

The composition of fungal inoculum and sterilized inoculum: The isolated AMF was multiplied with host plant *Zea mays L.* in a multi-spore pot culture containing a mixture of zeolite and expanded clay (1:1; v: v) for six months. Thus, the fungal inoculum comprised a mixture of spores, mycelium, zeolite, expanded clay, and plant root fragments. After the sterilization of fungal inoculum, the sterilized inoculum was obtained.

***Mycorrhizal assessment*:** AMF colonization in plant roots was measured before the addition of EOPs by the following procedure (Phillips and Hayman, 1970). Two grams of fresh roots were submerged in 10% KOH solution at 90 ℃ for 30 min, then washed by tap water, submerged in 2% HCl solution for 5 min, and stained with Trypan blue at 90 ℃ for 30 min, detained with 50% glycerol for 3 days. After that, the roots were cut into 1 cm fragments to check the mycorrhizal colonization under a stereomicroscope. The frequency of mycorrhiza in the root system (F%), the intensity of mycorrhizal colonization (M%) and arbuscular abundance (A%) in the whole root system was evaluated by the Mycocalc program (National Institute for Agricultural Research) (Trouvelot A; Kough, 1986).

Determination of MDA, $O_2^{\bullet-}$, POD, SOD, and soluble protein

Preparation of enzyme solution: Take 0.2g samples (fresh leaves or roots), wash and place in a pre-cooled mortar, add 1.6mL 50mmol/L pre-cooled phosphate buffer (pH 7.8). The solution was homogenized by grinding on an ice bath, transferred to a centrifuge tube and centrifuged at 12000 g for 20 min at 4 ° C, and the supernatant was an enzyme solution.

MDA: The level of lipid peroxidation was expressed as the content of MDA (Zhang et al., 2005). The fresh leaves or roots from each treatment were homogenized in 5 mL of 10 % trichloroacetic acid with a pestle and mortar. Homogenates were centrifuged at 4,000×g for 20 min. To each 2 mL aliquot of the supernatant, 2 mL of 0.6 % thiobarbituric acid in 10 %TCA was added. The mixtures were heated at 100 ℃ for 15 min and then quickly cooled in an ice bath. After centrifugation at 10,000×g for 20 min, the absorbance of the supernatant was recorded at 532 and 450 nm. Lipid peroxidation was expressed as the MDA content in nM per g FW.

POD: The POD activity was examined according to the modified method (Zhang et al., 2005). The reaction mixture in a total volume of 50 mL 0.1 mol/L sodium phosphate buffer (pH 6.0) containing 19 µL H_2O_2 (30 %) and 28 µL guaiacol was prepared immediately before use. Then, 1mL enzyme extract was added to 3 mL reaction mixture. An increase in absorbance was measured at 470 nm at 0.5 min intervals up to 2 min using a UV-Vis spectrophotometer. Enzyme specific activity is defined as units (one peroxidase activity unit defined as absorbance at 470 nm changes 0.1 per minute) per gram of fresh weight.

SOD: The SOD activity was detected according to the modified method (Zhang et al., 2005). The reaction mixture was made of 54 mL methionine, 2 mL nitro-blue tetrazolium chloride (NBT), 2 mL EDTA-Na_2, and 2 mL riboflavin. An appropriate quantity of enzyme extract was added to the reaction mixture. The reaction started by placing tubes below two 15 W fluorescent lamps for 15 min. Reaction stopped by keeping the tubes in dark for 10 min. Absorbance was recorded at 560 nm. One unit of SOD enzyme activity was defined as the quantity of SOD enzyme required to produce a 50 % inhibition of reduction of NBT under the experimental conditions, and the specific enzyme activity was expressed as units per gram fresh weight (FW) of leaf or roots.

$O_2^{\bullet-}$: Production of superoxide anion ($O_2^{\bullet-}$) was measured using the modified method (Hassoun and Ray, 2003). The reaction mixture contained 2 mL supernatant, 1.5 mL 50 0.05 mol/L phosphate-buffered saline (pH 7.8) and 1 mL 0.5 mmol/L hydroxylamine hydrochloride in the same buffer. After incubation for 20 min at 25 °C, the reactions were terminated by placing the reaction mixture tubes on ice. Then 4 mL of p-aminobenzene sulfonic acid and 0.4 mL of α-naphthylamine were added into 2.0 mL of the above reaction solution in sequence and place in a constant temperature water bath at 30 °C for 30 min, and measure A530 after the reaction.

Soluble protein: 5 mL 1.0 mL Coomassie Brilliant Blue Reagent was added into 1 mL enzyme solution. After shaking, it was placed for 5 min and measured A595.

Determination of EOPs and their metabolites

Extraction from soil and plant tissues:

The dry samples (0.5g fine powder) were extracted by 10mL of methanol: acetone (95:5, v/v) in a 12 mL vial in an ultrasonic bath for 30 mins. After extraction, the samples were centrifuged in a 50 mL conical polypropylene centrifuge vial (3000 rpm, 10 min). the supernatants were evaporated to dryness and reconstituted in 10% methanol, filtered through a 0.22 μm pore size polyvinylidene fluoride filter and stored at -20 °C until analysis.

Water sample preparation:

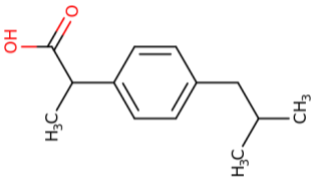
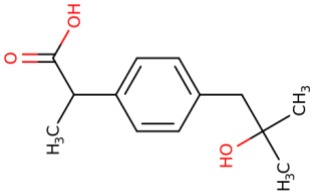
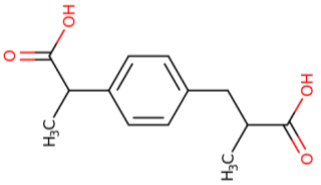
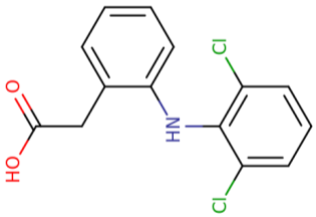
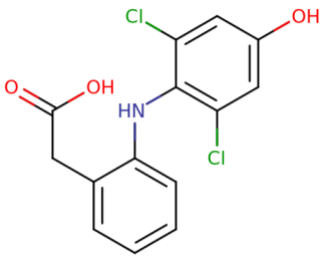
Before analysis, water samples should be filtered with a 0.22 μm pore size polyvinylidene fluoride filter.

Analysis：

A 1200 Ultra-High-Performance Liquid Chromatograph (UHPLC) tandem with 6410 Triple Quad Mass Spectrophotometer (MS/MS) of Agilent Technologies was used to analyze EOPs and its metabolites. The separation was carried out on a Water XBridge®-C-18 analytical column (150×4.6 mm, 3.5 μm particle size). The mobile phase consisted of methanol and water with 0.05% acetic acid as the mobile phase additive. The flow rate was 0.25 mL min/L. The injection volume was 1 mL. The surrogate recoveries were always higher than 90%. This method was modified from the previous study (Vymazal et al., 2017).

Tables:

Table S3.1. Physical-chemical capacities of EOPs and their metabolites.

EOPs	Structure	Molecular	$LogK_{ow}$[1]	pK_a[1]
Ibuprofen (IBU)		206.30	3.26	4
2-hyroxy ibuprofen (2-OH IBU)		222.28	4.15	4.63
Carboxy ibuprofen (CA IBU)		236.2637	2.78	3.97
Diclofenac (DCF)		296.15	4.26	4
4'-hyroxy diclofenac (4-OH DCF)		312.15	3.96	3.76

$LogK_{ow}$ and pKa (Strongest Acidic) values were collected from ChemAxon (https://chemaxon.com).

Table S3.2. The characteristics of simulated sewage.

Reagent	mg/L	Microelements	mg/L
Urea	104	$CuSO_4 \cdot 5H_2O$	0.01
NH_4Cl	16	$FeSO_4 \cdot 7H_2O$	0.45
$CH_3COONa \cdot 3H_2O$	255	$MnSO4 \cdot H_2O$	0.02
Peptone	20	$Pb(NO_3)_2$	0.02
KH_2PO_4	41	H_3BO_3	0.04
Yeast extract	132	$Na_2MoO_4 \cdot 2H_2O$	0.02
Skim milk	59	$KCr(SO_4)_2 \cdot 12H_2O$	0.02
$NaHCO_3$	25		
$MgSO_4 \cdot 7H_2O$	41		
$CaCl_2 \cdot 6H_2O$	28		

Modified according to the protocol (Nopens et al., 2001).

Table S3.3. The mean volume of each system effluent.

Treatments	Influent	Effluent	
		AMF-	AMF+
Replicate 1	2	1.27	1.12
Replicate 2	2	1.31	1.11
Replicate 3	2	1.29	1.13
Mean	2	1.29	1.12

Figures:

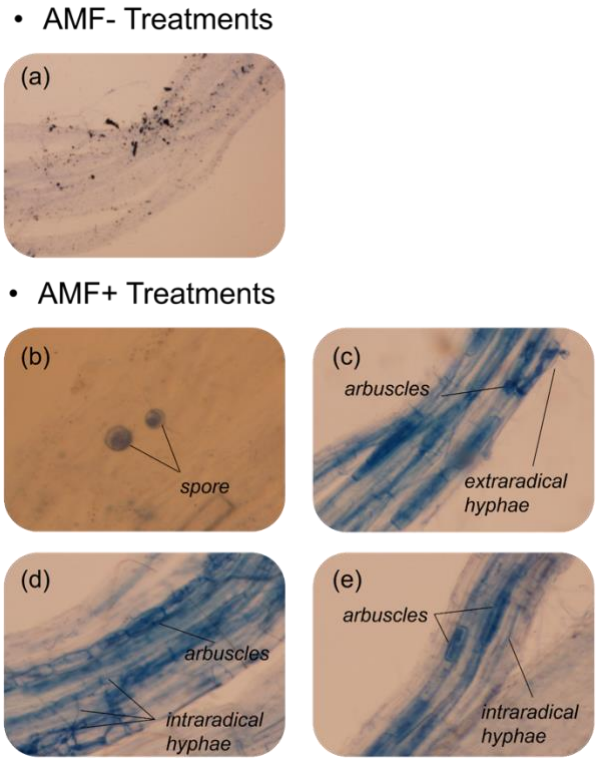


Fig. S3.1. AMF colonization in the roots of *G. maxima* in AMF- and AMF+ treatments.

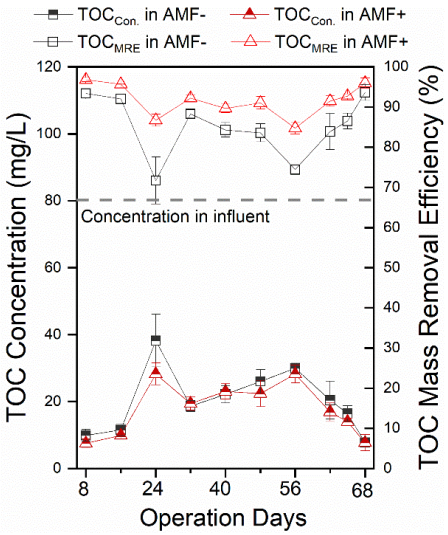


Fig. S3.2. Total organic carbon concentration ($TOC_{Con.}$) and its mass removal efficiency ($TOC_{MRE.}$) in AMF- and AMF+ treatments after the addition of 100% strength synthetic wastewater. The data are the means ± standard errors (n=3).

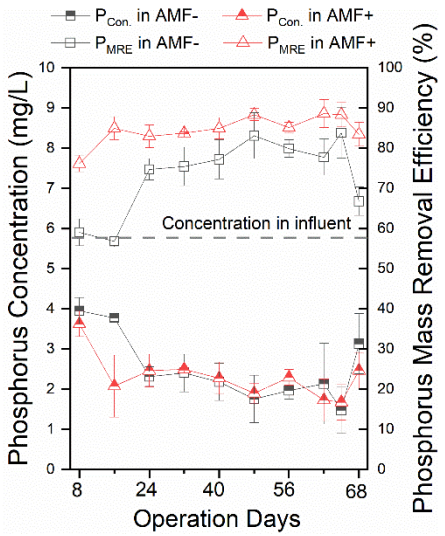


Fig. S3.3. Phosphorus ($P_{Con.}$) concentration and its mass removal efficiency ($P_{MRE.}$) in AMF- and AMF+ treatments after the addition of 100% strength synthetic wastewater. The data are the means ± standard errors (n=3).

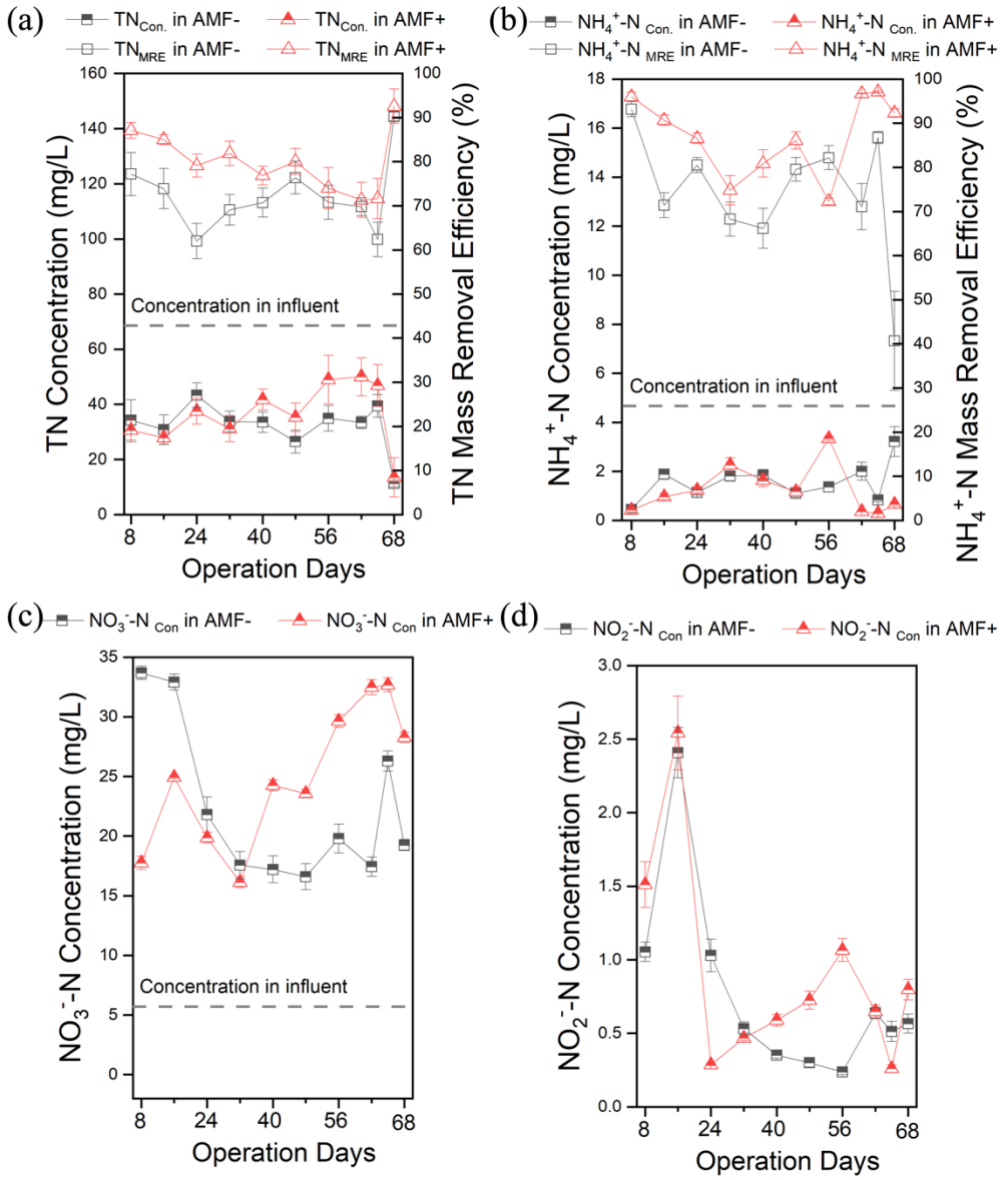


Fig. S3.4. (a) Total nitrogen concentration ($TN_{Con.}$) and its mass removal efficiency ($TN_{MRE.}$) in AMF- and AMF+ treatments. (b) NH_4^+-N concentration (NH_4^+-$N_{Con.}$) and its mass removal efficiency (NH_4^+-$N_{MRE.}$) in AMF- and AMF+ treatments. (c) NO_3^--N concentration (NO_3^--$N_{Con.}$) in AMF- and AMF+ treatments. (d) NO_2^--N concentration (NO_2^--$N_{Con.}$) in AMF- and AMF+ treatments, after the addition of 100% strength synthetic wastewater. The data are the means ± standard errors (n=3).

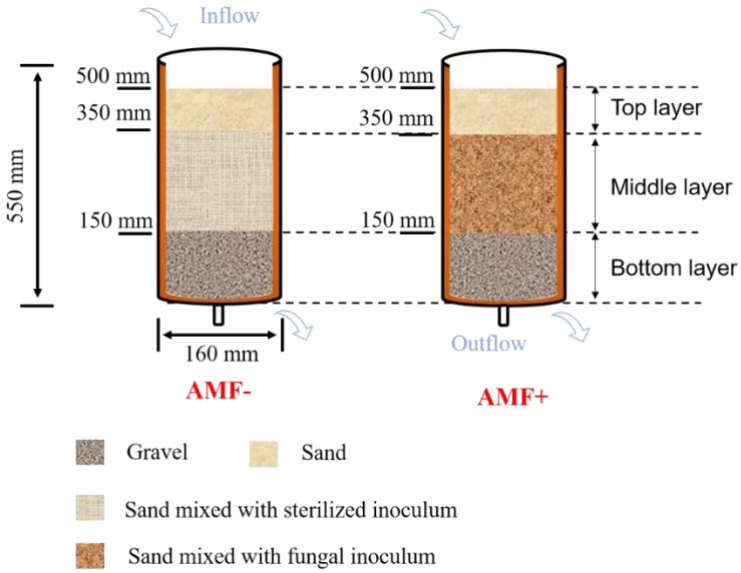


Fig. S3.5. Sketch of CWs reactors. The top layer and the middle layer in both AMF- and AMF+ treatments were the same, filled with sand and gravel, respectively. The middle layer in those two treatments is different, sand mixed with sterilized inoculum was added into the middle layer of AMF- treatments, and sand mixed with fungal inoculum was added into the middle layer of AMF+ treatments. Triplicates for each treatment.

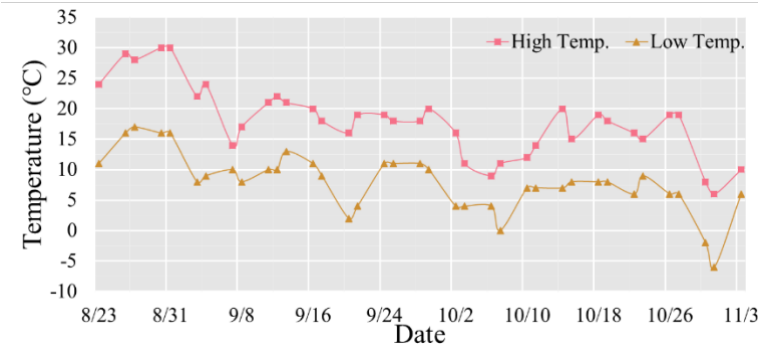


Fig. S3.6. The temperature (Temp.) changes during the experiment.

Chapter IV

Arbuscular mycorrhizal symbiosis in constructed wetlands with different substrates: effects on the phytoremediation of ibuprofen and diclofenac

Bo Hu, Shanshan Hu, Jan Vymazal, Zhongbing Chen

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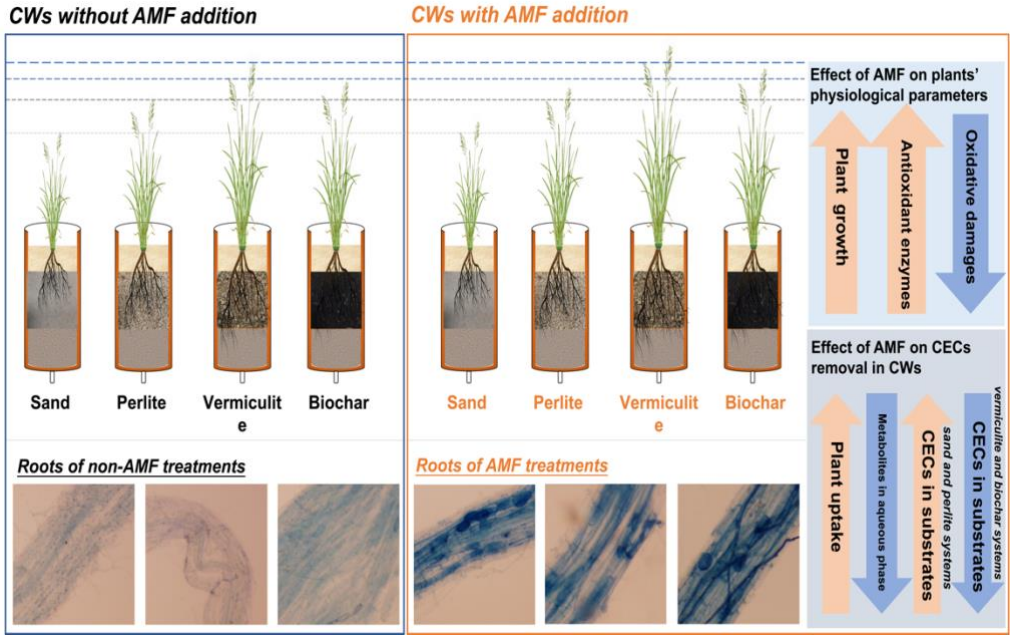
Contents

4.1 Abstract

This study investigated the role of arbuscular mycorrhizal fungal (AMF) for the removal of ibuprofen (IBU) and diclofenac (DCF) in constructed wetlands (CWs) with four different substrates. Results showed that AMF colonization in adsorptive substrate (perlite, vermiculite, and biochar) systems was higher than that in sand systems. AMF enhanced the tolerance of *Glyceria maxima* to the stress of IBU and DCF by promoting the activities of antioxidant enzymes (peroxidase and superoxide dismutase) and the contents of soluble protein, while decreasing the contents of malondialdehyde and $O_2^{\bullet-}$. The removal efficiencies of IBU and DCF were increased by 15%-18% and 25%-38% in adsorptive substrate systems compare to sand systems. Adsorptive substrates enhanced the accumulation of IBU and DCF in the rhizosphere and promoted the uptake of IBU and DCF by plant roots. AMF promoted the removal of IBU and DCF in sand systems but limited their reduction in adsorptive substrate systems. In all scenarios, the presence of AMF decreased the contents of EOPs metabolites (2-OH IBU, CA IBU, and 4'-OH IBU) in the effluents and promoted the uptake of IBU by plant roots. Therefore, these results indicated that the addition of adsorptive substrates could enhance the removal of IBU and DCF in CWs. The role of AMF on the removal of IBU and DCF was influenced by CW substrate. These may provide useful information for the application of AMF in CWs to remove emerging organic pollutants.

Graphical abstract:



4.2 Introduction

Emerging organic pollutants (EOPs) have been attracted increasing problems due to their potential adverse effects on ecosystems and human health. It has been reported that more than 1000 EOPs can be detected in the natural aquatic environment and most of them are not yet included in the monitoring list by many countries (NORMAN Association, 2016). As the degradation of EOPs in wastewater treatment plants (WWTPs) is often incomplete, the effluent of WWTPs has become the main source of EOPs in the aquatic environment (Farré et al., 2008). Consequently, the continuous discharge of EOPs into the environment and their potential biotoxicity to aquatic organisms has attracted increasing concerns. Constructed wetlands (CWs) are considered to be a sustainable technology for pollutant removal due to the lower cost, easy operation, and fewer maintenance requirements (Vymazal, 2014). Numerous studies documented that CWs showed advantages in removing EOPs (e.g., pharmaceuticals, pesticides, flame retardants, plasticizers, and surfactants) (Gorito et al., 2017). However, the potential impacts of EOPs and their metabolites on wetland plant growth and system stability have received increasing concerns owing to their wide occurrence in CW systems (Moro et al., 2014). For example, a study reported by Ziółkowska et al. (2014) suggested that EOPs negatively affect leguminous plants (lupin, pea and lentil), such as reducing seeding growth and change in enzymatic activity. Therefore, using CWs to treat EOPs may also bring new ecological concerns. There is an urgent need to improve the ability to remove EOPs in CW systems, and thus eliminating their potential negative impacts on ecosystems.

Arbuscular mycorrhizal fungi (AMF) are important components of the soil microorganisms, which can form symbiotic associations with the roots of most (nearly 85%) territorial plants (Ajit Varma, 2017; Smith and Read, 2008). AMF have positive effects on the performance of the host plant through increasing nutrient uptake and tolerance to environmental stresses, such as drought, salinity, heavy metals, and organic pollutions (Ajit Varma, 2017). Research has established that the presence of AMF can enhance the growth of ryegrass (*Loliu mperenne* L.) and white clover (*Trifolium mrepens* L.) in soil with 2000 mg/kg of twelve different polycyclic aromatic hydrocarbons (PAHs) (Joner and Leyval, 2003). Experimental evidences have been obtained for the contribution of AMF on removing EOPs in contaminated soil such as atrazine (Huang et al., 2007), PAHs (Gao et al., 2010), benzo [a] pyrene (Liu et al.,

2004). Meanwhile, Wu et al. (2008) demonstrated that AMF colonization (*Glomus etunicatum*) enhanced the uptake of dichlorodiphenyltrichloroethane (DDT) by the roots of alfalfa, and the symbiotic association significantly increased bacterial and fungal counts and dehydrogenase activity in the rhizosphere soil. These findings have indicated AMF might contribute to the removal of EOPs as a friendly and potentially biotechnological approach.

In the past two decades, a number of researchers have confirmed that AMF colonization was widely found in wetland plants from various habitats, including mangroves (Wang et al., 2015), fen and marshland (Bohrer et al., 2004), and urban wetland (Wang et al., 2018a). A review conducted by Xu et al. (2016), suggesting that plants of 99 families in 31 different wetland habitats have been found associated with AMF, even including submerged aquatic plants and several plant species that were thought to be nonmycorrhizal. However, there have been only a few reports on the application of AMF in constructed wetlands for EOPs or heavy metals contaminated wastewater bioremediation (Fester, 2013; Xu et al., 2018). Knowledge about the impacts of AMF in the removal of EOPs in wetland systems is still rare. Substrates, as an important component in CW systems, has been widely acknowledged to play a significant role in CWs for EOPs removal, by providing carries for biofilm development and medium for plant growth, and directly absorbing pollutant (Zhang et al., 2018). However, differences in CW substrates and their physiochemical properties have determining effects on the dissolved oxygen conditions, nutrients and water retention, which, in turn, influence the development and activities of microorganisms in CW systems (Yang et al., 2018). This indicated that substrates might also have potential impacts on the development of AMF in CWs (Xu et al., 2016). As far as we know, no previous research has investigated the effect of substrates on AMF colonization in CW systems and the application of AMF in different substrate wetland systems for EOPs bioremediation. Therefore, it is necessary to explore the impacts of AMF in eliminating EOPs from wastewater in mesocosm-CW systems with different substrates, and thus, which could provide a basis for the application of AMF in the bioremediation of EOPs in real-scale wetland systems.

Therefore, the aims of this study were to (1) assess AMF colonization in constructed wetlands with different substrates; (2) explore the role of AMF in the metabolism of EOPs in CWs with different substrates. The present research explores, for the first time, the effects of AMF colonization on the removal of EOPs in different substrate CWs and can provide new insight into the biodegradation of EOPs in CWs.

4.3 Materials and Methods

4.3.1 Chemicals and standards

Ibuprofen (IBU) and diclofenac (DCF) were used as the representative EOPs to assess the effects of AMF on the removal of EOPs in the present study, which are two common non-steroidal anti-inflammatory drugs with high consumption rates. They have been regarded as two of the most frequently detected EOPs in aquatic bodies (Madikizela and Chimuka, 2017) and wetland systems (Vymazal et al., 2017). The added concentrations of IBU and DCF in this study were 500 µg/L and 100 µg/L, respectively, which were close to their real concentrations in the influent of WWTPs (**Table S4.2, in the supporting information (SI)**). IBU and DCF with a purity > 98% were provided by Aldrich Chemical Co. Their physiochemical capacities were shown in **SI, Table S4.1**.

Rhizophagus irregularis (BEG 140, previously known as *Glomus intraradices*) is one of the most typical AMF species and frequently observed in the rhizosphere of wetland plants (Fester, 2013), which was selected as the AMF inoculum in this study. It was provided by the Institute of Botany, Czech Academy of Science (Prague, Czech Republic). AMF inoculum included mycorrhizal roots (*Zea mays L.*), spores, mycelium, and growth medium (zeolite and expanded clay, 1:1; v: v). The sterilized fungal inoculum was prepared by fungal inoculum at 120 ^{o}C for 2 h.

Glyceria maxima was selected as a host plant obtained from a local pond located in the Czech University of Life Sciences Prague. *G. maxima* is a common wetland species in Europe. Meanwhile, AMF colonization in the roots of *G. maxima* was also reported by previous studies (Hu et al., 2021a; Wang et al., 2018a).

Siliceous sand (sand), expanded perlite (perlite), expanded vermiculite (vermiculite), and biochar were selected as the CW substrate to design four different CW systems (**Fig. 4.1**). They were obtained from local companies in Prague, Czech Republic. Sand, perlite, vermiculite, and biochar showed different removal performances for EOPs in CWs, which have been reported by many studies (Abdelhakeem et al., 2016; Dordio et al., 2007; Yang et al., 2018). This could attribute to the differences in their physicochemical properties, such as particle size, porosity,

specific surface area, cation exchange capacity (Yang et al., 2018). Meanwhile, Sand, perlite, vermiculite, and biochar have different capacities to absorb EOPs: (1) No/low sorption: sand (Dordio et al., 2007); (2) Moderate sorption: perlite and vermiculite (Dordio et al., 2007; He et al., 2017); (3) High sorption: biochar (Brunsch et al., 2018). Therefore, these differences among the four substrates might also affect the functional roles of AMF in CWs for the removal of EOPs. **Fig. 4.2** showed the differences in the physicochemical properties of substrates.

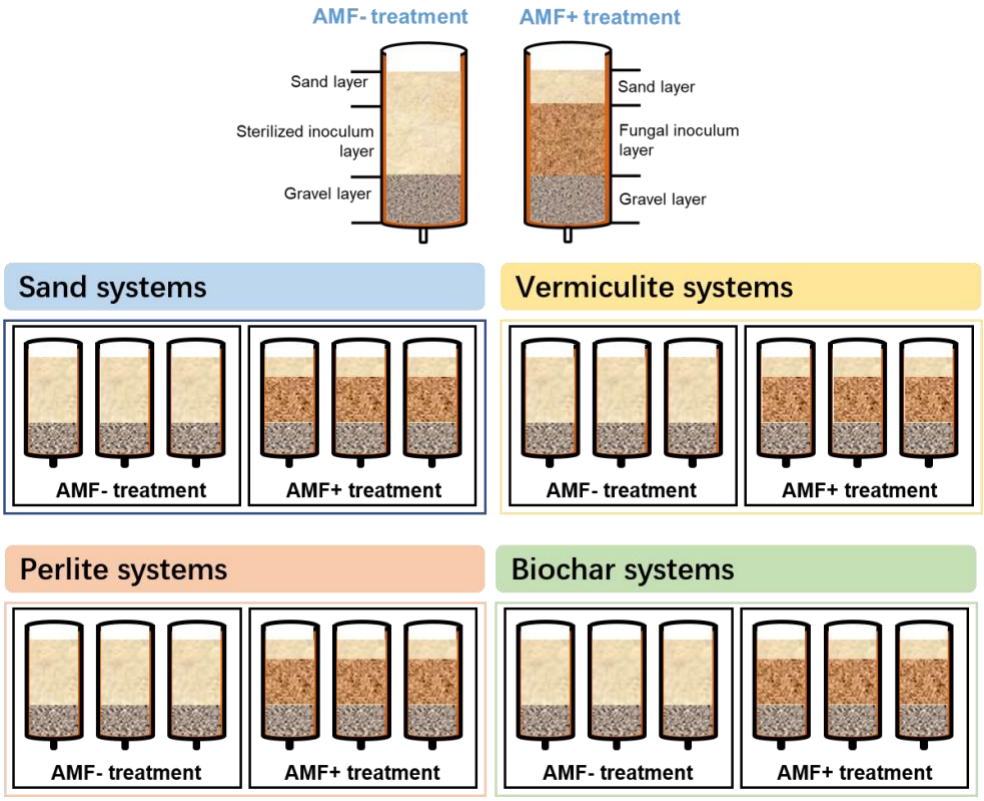


Fig. 4.1. Sketch of CWs systems. The composition of each column was 150 mm bottom layer filled with gravel, 200 mm middle layer filled with different substrates, and 150 mm top layer filled with sand. The middle layer of each system was filled with sand, perlite, vermiculite, and biochar, respectively. Two treatments were set: AMF+ treatments (and AMF- treatments. Each treatment has three replicates.

4.3.2 Experimental setup

Four CWs treatments filled with different substrates were designed in this study (**Fig. 4.1**). CW system was carried out in PVC-U materials columns with a diameter of 15 cm and a height of 55 cm. Each system was filled from bottom to top with 15 cm depth of gravel, 20 cm depth of substrates, and 15 cm depth of sand. The substrate layer was different in the sand, perlite, vermiculite, and biochar systems, which were filled with 20 cm depth of sand, perlite, vermiculite, and biochar, respectively. Before filled into the column, each substrate was sterilized at 120 ℃ for 3 h. Each substrate system was set into two treatments (AMF+ and AMF-). AMF+ treatments were added 350 g fungal inoculum and mixed with the middle layer substrates. For AMF- treatments, 350 g sterilized fungal inoculum together with 100 mL inoculum filtrate (Wu et al., 2018) were added into the middle layer substrates to provide a similar microflora except for the absence of AMF.

Two seedings of *G. maxima* (about 15 cm shoot height) were transplanted into the substrate layer of the system. Before transplantation, the roots of seedlings were washed carefully and sterilized (details were shown in **SI**). CW systems were fed with synthetic wastewater (Hu et al., 2021a). Previous studies indicated that the occurrence of AMF shows a negative correlation with water depth and duration of flooding (Mendoza et al., 2005; Miller, 2000; Wang et al., 2011). AMF colonization in wetland systems with an intermittent loading was significantly higher than that with continuously flooding (Liang et al., 2018b; Stevens et al., 2011). Hence, CWs operated with an intermittent loading (2 L/4 d, keep water for two hours and then drain for four days) during the whole experiment, which could be beneficial to the establishment of AMF symbiosis in wetland systems (Shi et al., 2015). In the first two months, CWs were fed with 10% strength synthetic wastewater to maintain the plant's growth and develop AMF in CW systems. Then CW systems were fed with EOPs-containing wastewater (100% strength synthetic wastewater mixed with 500 μg/L IBU and 100 μg/L DCF). The experiment was carried out in the natural environment with rain protection from July to November and ended after the temperature dropped below 0 ℃.

4.3.3 Sample analysis

Analysis of plants

AMF colonization in the roots of *G. maxima* was measured before the addition of ibuprofen and diclofenac, according to the methods described in our previous study (Hu et al., 2021a). At the end of the experiment, all plants (with an intact root systems) were carefully removed from CW systems, washed carefully with deionized water, then divided into shoots (aboveground) parts and roots (underground) parts. The growth of *G. maxima* (root length, shoot height) was measured by fresh plants. Meanwhile, the content of malondialdehyde (MDA), superoxide anion ($O_2^{\bullet-}$), antioxidant enzymes (peroxidase (POD) and superoxide dismutase (SOD)), and soluble protein was analyzed by using the fresh sample of plant's tissues. The detailed methods for the measurement of those physiological parameters were shown in our previous study (Hu et al., 2021a). The biomass of *G. maxima* (root and shoot weight) was measured by dry plant samples.

Measurement of EOPs

The distribution of parent EOPs (IBU and DCF) and their metabolites (carboxy ibuprofen (CA IBU), 2-hydroxy ibuprofen (2-OH IBU), and 4'-hydroxy diclofenac (4'OH DCF)) in CW systems were analyzed by liquid chromatography-mass spectrometry (LC-MS).

After the addition of EOPs, water samples of the influent and effluent were taken every 12 days. Water samples were taken in 60 mL amber glass vials and stored in a freezer (-28 ^{o}C). The measurement of EOPs and their metabolites in the water sample were carried out immediately after defrosting. Moreover, the accumulation of parent EOPs and their metabolites in plant tissues (roots and shoots) and the rhizosphere substrates were analyzed at the end of this experiment. The detailed analysis procedures were shown in our previous study (Hu et al., 2021a).

4.3.4 Data analysis

Owing to the evaporation and transpiration in CW systems, the volume of the effluent was lower than that of the influent (**SI, Table S4.3**). Therefore, the removal efficiency of EOPs was evaluated by calculating their mass removal rate (Eq. (1)). V_{In} and V_E are the volumes of the influent and effluent, C_{In} and C_E are the concentration of EOPs in the influent and effluent.

$$\text{Mass removal efficiency (\%)} = \frac{V_{In} \times C_{In} - V_E \times C_E}{V_{In} \times C_{In}} \times 100 \qquad (1)$$

Data are presented as mean and standard errors from data of the three replicates treatments. Physiological parameters of plants, such as MDA, $O_2^{\bullet-}$, POD, SOD, soluble protein, and the content of parent EOPs and their metabolites were analyzed using two-way analysis of variance (ANOVA), with substrates and AMF treatments as main factors and substrates * AMF treatments as an interaction effect. The student's t-test was used to compare the effect of AMF on physiological parameters of plants, the content of parent EOPs and their metabolites. Significant differences and extremely significant differences were set as $p < 0.05$ and $p < 0.01$, respectively. R Software (version 4.0.2) was used for statistical analysis and the visualization of experimental data.

4.4 Results and discussion

4.4.1 AMF colonization in different substrate systems

AMF colonization in the roots of *G. maxima* was observed from different substrate systems. The mycorrhizal frequencies in the roots of *G. maxima* were ranged from 61.3% to 92.3% (**Table 4.1**). Mycorrhizal structures were observed clearly in the roots of *G. maxima* from different substrate systems (**SI, Fig. S4.1**). These results indicated that the symbiosis between AMF and *G. maxima* could be established in different substrate systems under intermittent loading. This could mainly due to the aerenchyma structure in wetland plants that can provide active ventilation of roots, rhizomes, and the nearby rhizosphere, which maintains favorable oxygen conditions for AMF growth

(Dickopp et al., 2011). Meanwhile, the intermittent loading in the present study might also promote the oxygenation of the rhizosphere during the exchange of wet and dry periods, which could contribute to the establishment of symbiosis by providing suitable oxygen in CW systems. This was in good agreement with Miller (2000) who found that the roots of plants (*Panicum hemitomon* and *Leersia hexandra*) in CW under intermittent flood conditions had higher mycorrhizal colonization than those under continuous flood condition.

Table 4.1. Mycorrhizal status under different treatments. These include the frequency of mycorrhiza in the root system (F%), the intensity of mycorrhizal colonization (M%), and the arbuscule abundance (A%) in the whole root system. Data are presented as means ± standard errors. a, b, and c show a significant difference ($p < 0.05$).

Substrates	F%		M%		A%	
	AMF-	AMF+	AMF-	AMF+	AMF-	AMF+
Sand	0	61.34±10.48^{a}	0	11.24±2.47^{a}	0	0.54±0.07^{a}
Perlite	0	83.54±5.63^{bc}	0	26.23±1.34^{c}	0	0.79±0.04^{c}
Vermiculite	0	92.27±3.57^{b}	0	58.17±0.56^{b}	0	1.32±0.02^{b}
Biochar	0	78.36±6.34^{bc}	0	24.43±1.62^{c}	0	0.67±0.05^{c}

Moreover, the addition of adsorptive substrates (perlite, vermiculite, and biochar) showed positive effects on promoting AMF colonization in CW systems ($p < 0.05$) (**Table 4.1**). In adsorptive substrate systems, mycorrhizal status in the roots of *G. maxima* was significantly higher than that in sand systems ($p < 0.05$), in which the mycorrhizal frequency (F%), mycorrhizal colonization (M%), and arbuscule abundance (A%) were increased by 17.0%-31.0%, 13.2%-46.9%, and 0.1%-0.8%, respectively. The best mycorrhizal status was detected in vermiculite systems, in which F%, M%, and A% were 92.3%, 58.2%, and 1.3%, respectively. A similar mycorrhizal status was found in perlite and biochar systems. These results suggested that the symbiosis between AMF and *G. maxima* was affected by CW's substrates. The higher AMF colonization could be partly due to well-developed plant root systems (**SI, Table S4.4**), which can release more oxygen into the rhizosphere to promote the establishment of symbiosis (Xu et al., 2016). Compared with sand systems, the addition of perlite, vermiculite, and biochar alter the physical properties inside the CW systems (**Fig. 4.2**),

such as bulk density, porosity, and water storage capacity, thus could increase the retention of nutrients and water (**SI, Table S4.3**), which significantly affected the growth of *G. maxima* (**SI, Table S4.4**). Meanwhile, the essential elements in perlite, vermiculite and biochar, such as C, K, and Mg (**Fig. 4.2**), might also act as nutrient sources to improve plant growth and AMF development in CWs. Moreover, vermiculite and biochar could retain more nutrients inside the CW systems to promote plant growth and AMF development due to the higher specific surface area and cation exchange capacity (Yang et al., 2018). Besides, the larger surface area provided by the porous structure of biochar and the layered structure of vermiculite, as shown in (**Fig. 4.2**), could be considered a perfect bio-carrier for AMF development to increase the density of viable hyphae (Wen et al., 2016). However, AMF colonization in biochar systems was lower than that of perlite and vermiculite systems. The possible reason may be that the formation of harmful substances in biochar during its manufactures, such as dioxin, ethylene, polycyclic aromatic hydrocarbons, phenolic compounds, volatile compounds, and heavy metals (Lehmann and Joseph, 2012), could pose negative and inhibitory impacts on plant growth and AMF development. Therefore, the formation of AMF symbiosis in CW systems can be influenced by CW substrates.

4.4.2 Physiological parameters in wetland plants

As one of the main reactive oxygen species, $O_2^{\bullet-}$ accumulation can induce lipid peroxidation of plants, while MDA determines the level of lipid peroxidation of plants caused by oxidative stress (Zhang et al., 2019). Hence, the content of $O_2^{\bullet-}$ and MDA is related to the oxidative damage of plants. POD and SOD can trigger an antioxidative response to convert ROS into non-toxic molecules (Zhang et al., 2019). Therefore, the growth status of plants under the stress of IBU and DCF in different substrate systems can be obtained by analyzing the differences in physiological parameters of *G. maxima*.

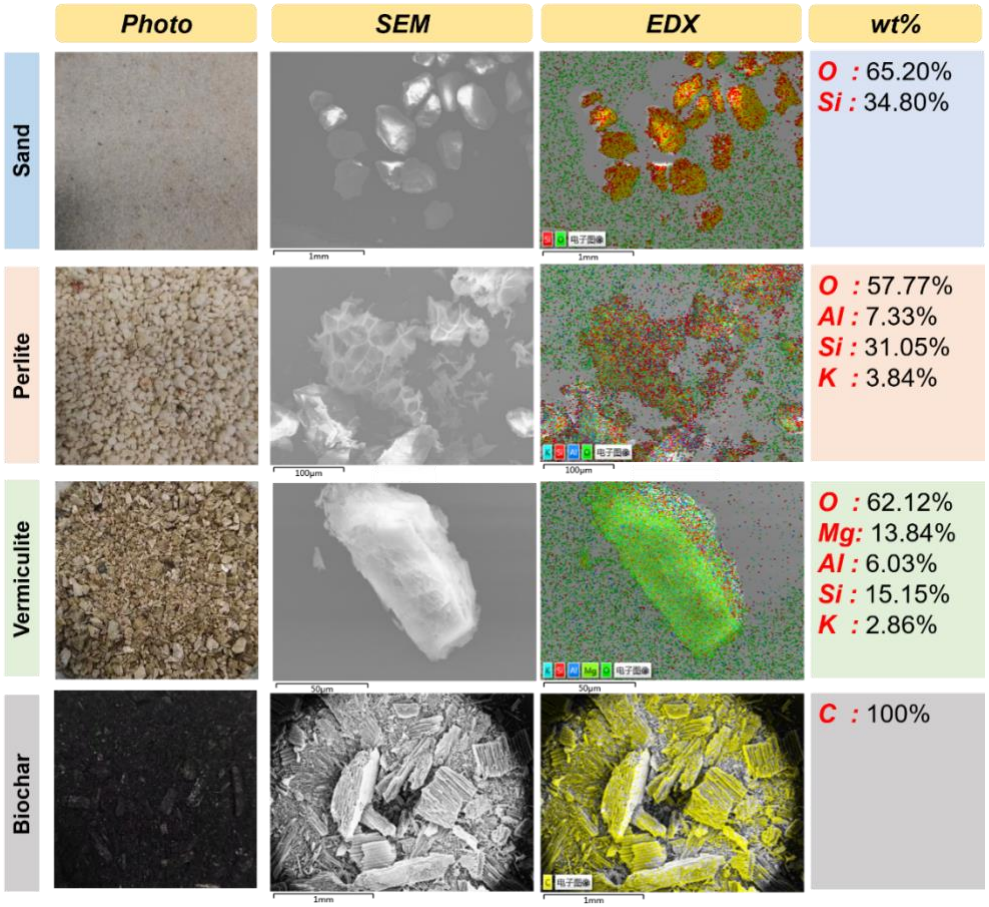


Fig. 4.2. SEM images and EDX spectra of sand, vermiculite, perlite, and biochar. Silica sand is made up of two main elements: Si and O, which is a chemically inert and relatively hard mineral. Perlite is a glassy volcanic rock, which is essentially a metastable amorphous aluminum. Expanded perlite likes little balls with a soft honeycomb texture inside. The basic elements in perlite include O, Al, Si, and K. Vermiculite have a highly porous and spongy structure. The basic elements of vermiculite include O, Mg, Al, Si, and K. Biochar shows a porous structure with a high surface area. Carbon is the main element in biochar. These results were obtained by SEM-EDX (FEI QUANTA FEG 650 combined with EDAX Inc. Genesis XM).

Effect of substrate on physiological parameters of plants

CW substrates had an extremely significant effect ($p < 0.001$) on the physiological parameters of *G. maxima* (**SI, Table S4.5**). Compared with sand systems, the contents of POD, SOD, MDA, $O_2^{\bullet-}$, and soluble protein in plant tissue (both roots and shoots) increased in biochar systems but decreased in perlite and vermiculite systems (**Fig. 4.3, SI, Fig S4.2, and S4.3**). Moreover, the content of those physiological parameters in plant tissue was the lowest in vermiculite systems. These results indicated that CW substrates could affect the physiological functions of wetland plants in CWs.

Substrates influenced the physiological parameters of *G. maxima* in CWs under the stress of IBU and DCF. The main reason may be that the type of substrates changes the operating conditions inside the CWs, which directly influences plant growth and EOPs removal. Compared with sand, the porous structure of vermiculite and perlite could increase the retention of available nutrients and water (**SI, Table S4.3**), which significantly promotes plant growth (**SI, Table S4.4**), thereby enhancing the removal of EOPs (Dordio et al., 2009). However, the oxidative damage of *G. maxima* in biochar systems was higher than that in sand systems. This could attribute to the potential harm of biochar to plant growth. A study conducted by Easton et al. (2015) suggested that the addition of biochar in CW systems significantly promoted the microbiological reduction of SO_4^{2-} and increased the generation of harmful by-products, including H_2S methyl mercury, and thus inhibit the formation of the symbiotic structures and plant growth. Besides, our study also found that the accumulation of IBU and DCF in the rhizosphere and *G. maxima* roots from biochar systems was significantly higher ($p < 0.05$) than that from other systems (**Table 4.2**). This indicates that the negative effect of biochar on AMF colonization is probably attributed to the high contents of IBU and DCF in the rhizosphere accumulated by biochar. Therefore, the tolerance of plants to EOPs stress could be influence by CW substrates.

Table 2. The content of ibuprofen, diclofenac and their metabolites in the rhizosphere soil and plant tissue (shoots and roots).

Substrate	Ibuprofen		2-hydroxy Ibuprofen		Carboxy Ibuprofen		Diclofenac		4'-hydroxy Diclofenac	
	AMF-	AMF+	AMF-	AMF+	AMF-	AMF+	AMF-	AMF+	AMF-	AMF+
Rhizosphere soil										
Sand	n.d.	111.0±5.97	n.d.	45.1±2.82	n.d.	20.2±0.54	n.d.	33.4±1.52	n.d.	n.d.
Perlite	11.3±0.79	164.0±9.18*	n.d.	37.9±0.11	n.d.	11.0±0.82	n.d.	46.4±0.03	n.d.	n.d.
Vermiculite	76.1±1.15	15.5±0.89*	54.9±4.74	n.d.	11.2±0.84	n.d.	30.9±1.00	n.d.	n.d.	n.d.
Biochar	129.0±3.31	104.0±4.53*	36.2±2.81	n.d.	14.2±0.24	n.d.	33.6±1.67	n.d.	n.d.	n.d.
Roots										
Sand	69.7±5.58	92.8±6.55*	155.0±9.11	104.0±7.69*	27.9±0.41	18.1±0.46*	61.7±1.61	150.0±0.17*	65.2±4.17	163.0±7.00*
Perlite	68.2±4.83	181.0±0.71*	171.0±5.09	274.0±1.06*	16.1±1.39	39.8±2.23*	98.0±4.89	187.0±15.94*	75.3±3.39	226.0±12.53*
Vermiculite	77.5±2.43	183.0±2.22*	59.3±3.49	128.0±6.99*	13.5±0.22	n.d.	128.0±9.88	47.0±2.56*	105.0±3.08	n.d.
Biochar	112±2.83	166.0±12.87*	86.3±6.26	123.0±8.56*	15.5±1.04	n.d.	206±0.24	27.3±0.47*	96.9±0.72	n.d.
Shoots										
Sand	10.64	n.d.	82.1±2.15	73.4±0.53*	n.d.	n.d.	10.5±0.44	n.d.	n.d.	n.d.
Perlite	10.9±0.32	19.8±0.52*	95.4±0.72	202.0±6.32*	n.d.	n.d.	11.5±0.82	24.8±0.63*	n.d.	n.d.
Vermiculite	13.1±0.61	124.0±3.51*	71.3±3.06	51.5±2.33*	n.d.	n.d.	13.1±0.86	59.3±3.13*	n.d.	n.d.
Biochar	n.d.	83.2±6.72	33.1±2.64	76.4±3.87*	n.d.	n.d.	n.d.	44.3±2.56	n.d.	n.d.

n.d.: not detected, below the detection limit (<10 μg/kg). Unit: μg/kg.

Effect of AMF on physiological parameters of plants

MDA and $O_2^{\bullet-}$ contents in inoculated plant roots under AMF+ treatments were significantly lower ($p < 0.05$) than those under AMF- treatments, with an increase of 19.4%-75.3%, 17.5%-27.3%, respectively (**Fig. 4.3**). On the contrary, the contents of POD and SOD in *G. maxima* roots from inoculated CWs were increased, which were 0.5%-83.5%, 16.5%-37.5% higher than those from non-inoculated CWs, respectively. Similar results were also observed in the shoots of *G. maxima*, the contents of MDA and $O_2^{\bullet-}$ were decreased, but the contents of POD, SOD, and protein were increased in AMF+ treatments (**SI, Fig. S4.2**). Moreover, AMF significantly increased ($p < 0.01$) the content of soluble protein in the tissues (both shoots and roots) of *G. maxima* (SI, Fig. S3). According to the results of two-way ANOVA analysis (**SI, Table S4.5**), AMF had an extremely significant effect ($p < 0.001$) on the physiological parameters of *G. maxima*, including MDA, $O_2^{\bullet-}$, POD, SOD, and protein.

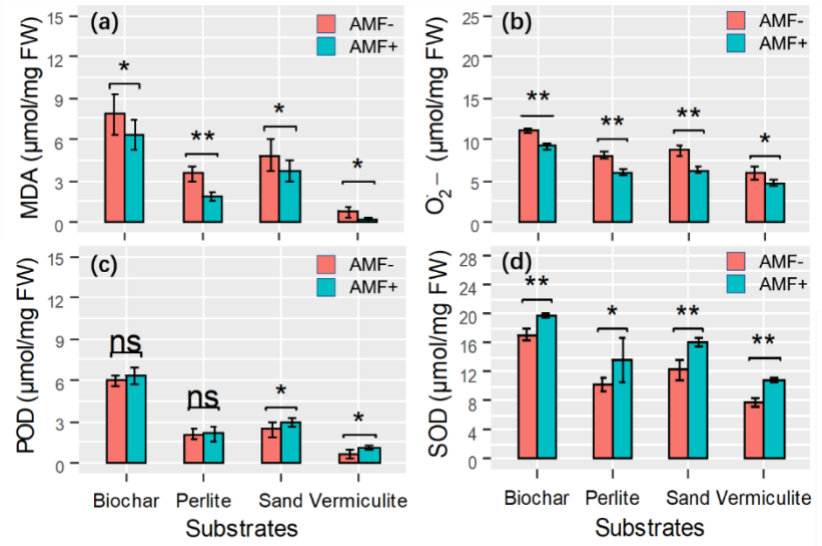


Fig. 4.3. Physiological parameters of plant roots: (a) MDA, (b) $O_2^{\bullet-}$, (c) POD, (d) SOD. The data are the means ± standard errors (n=3). * and ** show the significant difference ($p < 0.05$) and extremely significant difference ($p < 0.01$), respectively, in AMF- and AMF+ treatments. MDA: malondialdehyde; $O_2^{\bullet-}$: superoxide anion; POD: peroxidase; SOD: superoxide dismutase.

AMF can maintain ROS homeostasis to protect the host plant from various abiotic stresses (Zhang et al., 2019). In this study, AMF showed positive effects on increasing the activities of antioxidant enzymes (POD and SOD) and protein content as well as decreasing ROS ($O_2^{\bullet-}$) accumulation and lipid peroxidation (MDA), indicating that AMF could alleviate or reduce oxidative injuries of *G. maxima* conducted by the stress of IBU and DCF in different substrate CW systems. The possible reason could be that AMF facilitates the acquisition of nutrients and water uptake, which are beneficial to promote the host plant growth (**SI, Table S4.4**), and thus alleviating the adverse effects of abiotic stress on the host plants (Hu et al., 2020b). Another reason may be that AMF could regulate the expression of genes related to the synthesis of antioxidant enzymes. Existing evidence suggests that 30 genes potentially encoding for antioxidant enzymes were recorded in the genome of *R. irregularis* (Lenoir et al., 2016). Zhang et al. (2019) studied the effect of AMF on ROS generation and ROS scavenging ability under Pb stress and found that AMF symbiosis might maintain ROS homeostasis by down-regulating the transcription of MtRbohC-G to decreased the H_2O_2 accumulation, by inhibiting ROS generation induced by Pb stress, and by stimulating antioxidant response to scavenging redundant ROS. In their study, the activities of antioxidant enzymes in AMF inoculated *Medicago truncatula* were increased 43-322%, while the contents of MDA and $O_2^{\bullet-}$ were decreased 18-38% and 20-38%, respectively compared to non-inoculated controls under Pb stress. Similarly, Wu et al. (2020) confirmed that AMF could play an essential role in relieving the photosynthesis inhibition of host plants conducted by abiotic stress through up-regulating the expression of photosynthesis-related genes. Like our study, Aranda et al. (2009) indicated that AMF could promote protein secretion (glycoproteins) to improve nutrient uptake by host plants. In addition, by analyzing the physiological parameters of plant roots and the content of EOPs in plant roots, the result of cluster analysis showed that two distinct groups were observed in all substrate systems, namely AMF- and AMF+ treatments (**Fig. 4.4**). AMF showed a significantly positive effect on increasing POD, SOD, and protein contents but decreasing the contents of MDA and $O_2^{\bullet-}$ in *G. maxima*, suggesting that AMF may play important roles in plant detoxification. Therefore, AMF could protect wetland plants from EOPs stress in CW systems by hormonal regulation.

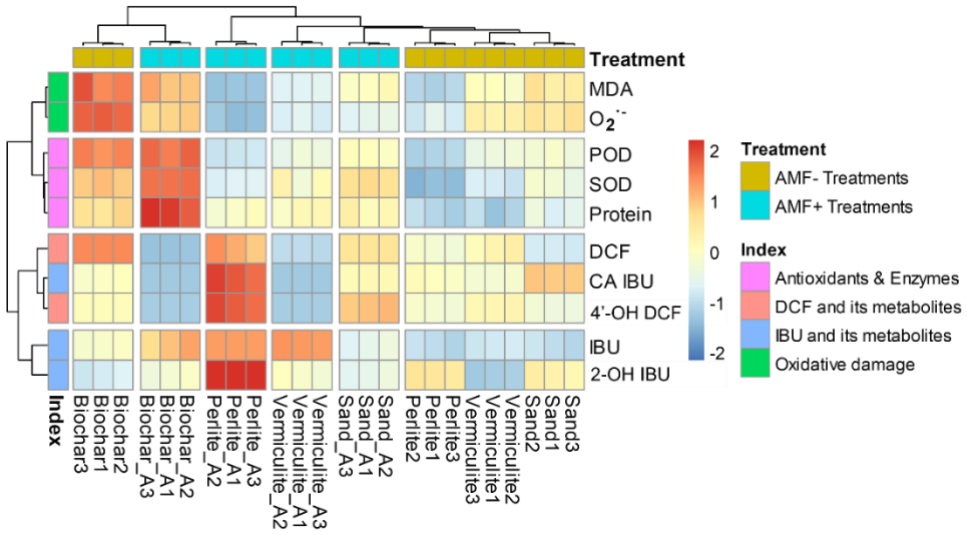


Fig. 4.4. The cluster analysis among physiological parameters in plant roots, the content of parent EOPs, and their metabolites in plant roots. Colors in the heatmap indicate the correlation between the different data sets. A represents AMF. 1, 2, and 3 represent the replicate of each treatment. MDA: malondialdehyde; $O_2^{\bullet-}$: superoxide anion; POD: peroxidase; SOD: superoxide dismutase; IBU: ibuprofen; DCF: diclofenac; 2-OH IBU: 2-hydroxy ibuprofen; CA IBU: carboxy ibuprofen; 4'-OH DCF: 4'-hydroxy diclofenac.

4.4.3 Effect of substrates on EOPs removal

The removal efficiencies of IBU and DCF in different CW systems were in the following order: sand < perlite < vermiculite < biochar. Compared with sand systems, the removal efficiencies of IBU and DCF were significantly increased by 14.6%-18.0% and 24.5%-38.2%, respectively, in perlite, vermiculite, and biochar systems (**SI, Fig. S4.4**). The concentrations of parent EOPs (IBU and DCF) and metabolites (2-OH IBU, CA IBU, and 4'-OH DCF) were the highest in the effluent from sand systems (**Fig. 4.5 and 4.6**), in which the removal rate of IBU and DCF were 87% and 61%, respectively (**SI, Fig. S4.4**). In biochar systems, the mass removal rates of IBU and DCF were the highest (up to 99%) (**SI, Fig. S4.4**) and the concentrations of parent EOPs and metabolites in the effluent were decreased below the detection limit (< 0.002 ug/L) (**Fig. 4.5 and 4.6**). The removal efficiencies of IBU and DCF in vermiculite systems were 1.1% and 0.5% higher than those in perlite systems (**SI, Fig. S4.4**), and no

significant differences in the removal of IBU and DCF were found between perlite and vermiculite systems under AMF- treatments (**SI, Table S4.7**). Meanwhile, their metabolites in the effluent from vermiculite systems were higher than those from perlite systems (**Fig. 4.6**). The results of two-way ANOVA analysis indicated that substrates had a significant effect ($p < 0.001$) on the concentrations of both parent EOPs and metabolites in the effluent of CWs (**SI, Table S4.6**).

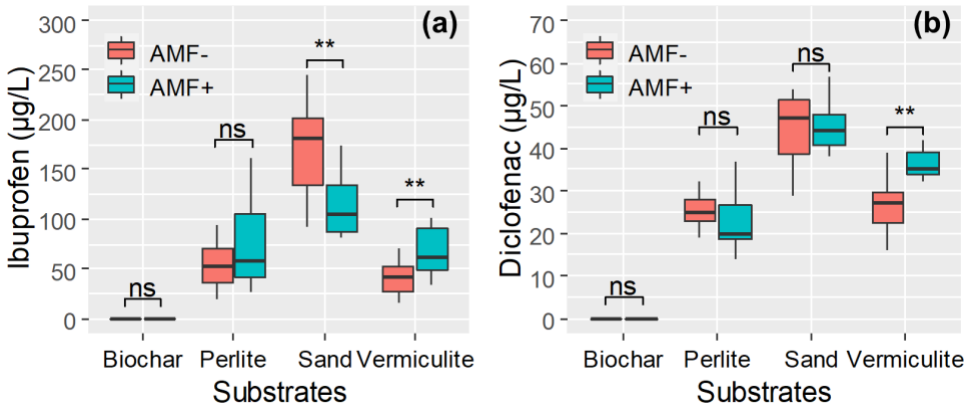


Fig. 4.5. The concentrations of parent EOPs in the outflow of different CW systems: (a) ibuprofen, (b) diclofenac. The data are the means ± standard errors (n=15). ns and ** show the no significant difference ($p > 0.05$) and extremely significant difference ($p < 0.01$), respectively, in AMF- and AMF+ treatments.

CW substrates can influence the removal performance of EOPs in CWs. The main reason is that type of substrates could influence the establishment and growth of biofilm and microbial community, which can directly affect the biodegradation of EOPs in CWs (Zhang et al., 2018). In this study, the addition of perlite, vermiculite, and biochar significantly ($p < 0.05$) increased the removal efficiency of IBU and DCF compared with sand systems (**Fig. 4.5**), mainly facilitating the biodegradation of CECs in CWs (Deng et al., 2021). The porous structures and large surface area of perlite, vermiculite, and biochar could be the excellent attachment carrier for CEC-degrading microbes, which promoted the development of microorganisms in the rhizosphere (Ilyas and Van Hullebusch, 2020). Meanwhile, the addition of perlite, vermiculite, and biochar in CWs improved the growth of *G. maxima* (both shoots and roots) (**SI, Table S4.4**), which might also promote radial oxygen loss and organic exudate secretion, thus enhancing the aerobic microbial metabolism of conventional pollutants and EOPs (Deng et al., 2021). Previous studies suggested that the metabolites of IBU and DCF, including 2-

OH IBU, CA IBU, and 4'-OH DCF, are occurring under aerobic conditions (Bouju et al., 2016; Zwiener et al., 2002). The better removal performance of IBU and DCF in vermiculite systems than perlite systems might also relate to its more developed plant root systems (**SI, Table S4.4**), which could accelerate the removal of IBU and DCF through the aerobic metabolic pathway.

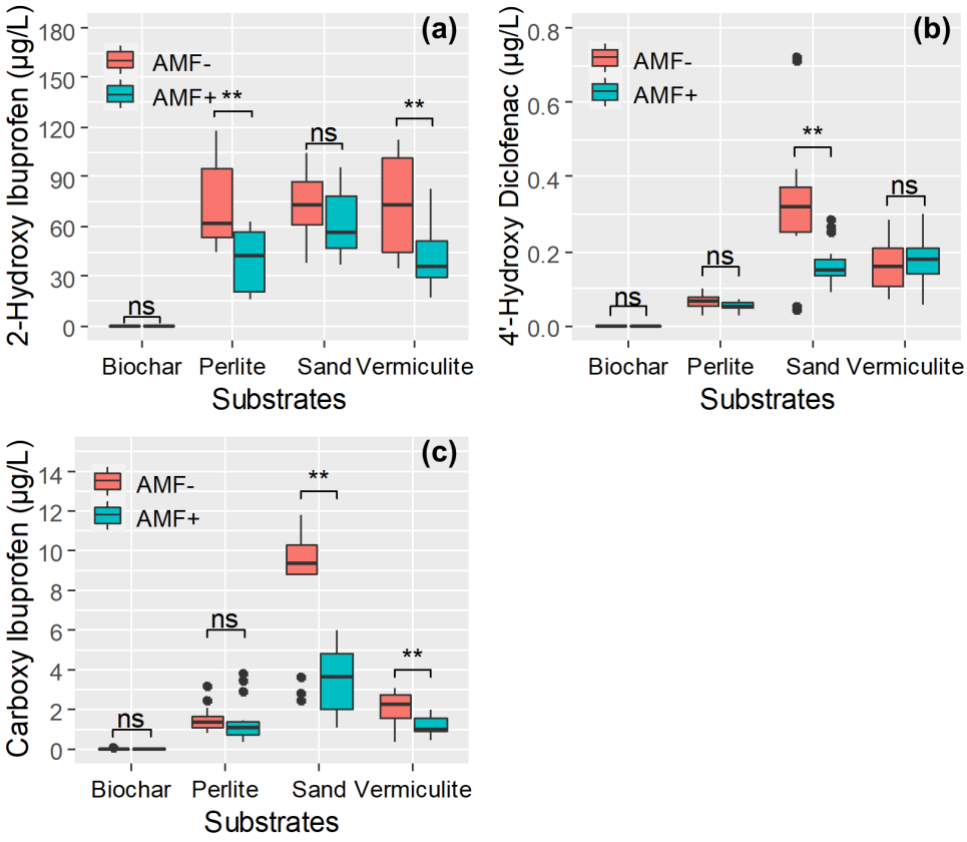


Fig. 4.6. The concentrations of EOPs metabolites in the outflow of different CW systems: (a) 2-Hydroxy ibuprofen, (b) Carboxy ibuprofen, and (c) 4'-Hydroxy diclofenac. The data are the means ± standard errors (n=15). ns and ** show the no significant difference ($p > 0.05$) and extremely significant difference ($p < 0.01$), respectively, in AMF- and AMF+ treatments.

Moreover, another reason for the positive effects of perlite, vermiculite, and biochar on the removal of IBU and DCF in CW systems could be that their adsorption of EOPs can play important roles in promoting the removal of EOPs. As shown in **Table 2**, the accumulations of IBU and DCF in the rhizosphere of different substrate systems mainly were in the order: sand < perlite < vermiculite < biochar (**Table 4.2**), indicating that the removal efficiencies of IBU and DCF in different substrate systems were highly related to the adsorption capacity of CW substrate to EOPs. These results agreed with previous studies (Liu et al., 2021). Their adsorption capacity mainly determined the removal of oxytetracycline and ciprofloxacin in different substrates (zeolite, gravel, red brick, and oyster shell) systems.

Furthermore, substrates also influenced the uptake of EOPs by plant roots. It was found that the contents of IBU and DCF in *G. maxima* roots followed the order: sand < perlite < vermiculite < biochar, suggesting that the application of adsorptive substrates could enhance the uptake of EOPs by plant roots (**Table 4.2**). Since most EOPs are xenobiotics, there are no specific transporters for these compounds in plant tissue. Diffusion (passive uptake) is the primary way for EOPs to enter the plant interior through the cells and membranes (Stottmeister et al., 2003). Therefore, the ability of the plant to take up EOPs may depend on the concentrations of EOPs in the rhizosphere, which was highly related to the amount of EOPs adsorbed by substrates (**Table 4.2**). Besides, substrates showed a significant difference in the partition of EOPs in plant tissue, such as the distribution of IBU and DCF in both roots and shoots ($p < 0.01$), the translocation of metabolites in roots ($p < 0.001$), and the translocation of 2-OH IBU in shoots ($p < 0.001$). However, the present results still cannot reveal the underlying mechanism of these differences. Future research needs to focus on the direct effects of plants on EOPs, such as the metabolic process of EOPs in plants, and the detoxification capacity of plants to EOPs.

4.4.4 Effect of AMF on EOPs removal in different substrate systems

The removal of EOPs in the liquid phase

The removal performance of IBU and DCF in the same substrate CW systems was different (**Fig. 4.5**). In sand systems, AMF significantly increased the removal of IBU

by 6.5% ($p < 0.01$) but slightly enhanced the removal of DCF by 0.1% ($p > 0.05$) (**Fig. 4.5 and SI, Fig. S4.4**). In vermiculite systems, the removal efficiencies of IBU and DCF were decreased by 1.6% and 3.4% in AMF+ treatments compared to AMF- treatments. However, the concentration of IBU and DCF in the effluent of perlite systems had no difference between AMF+ and AMF- treatments ($p > 0.05$). Meanwhile, since the low concentration of IBU, DCF, and their metabolites in the effluent from biochar systems, no difference in the effect of AMF on EOPs removal can be observed. Besides, a lower concentration of EOPs metabolites (2-OH IBU, CA IBU, and 4'-OH DCF) was detected in the effluent of AMF+ treatments than that in AMF- treatments. In addition, the results of two-way ANOVA showed that AMF had a significant effect on the concentrations of CECs, such as DCF, 2-OH IBU, and CA IBU, and the interaction effect between AMF and substrates had a significant impact on EOPs concentrations ($p < 0.05$) (SI, Table S7). Significant differences in the removal of IBU and DCF were shown among the four substrate systems under AMF+ treatments ($p < 0.001$) except that there was no difference in the removal of IBU between vermiculite and perlite systems (**SI, Table S4.7**).

AMF in sand systems significantly improved the removal of IBU but showed little effect on the removal of DCF. This may be because AMF is easier to promote the removal of EOPs in the aerobic metabolic pathway. AMF can enhance the removal of EOPs because it can promote plant growth and reduce the adverse effect of EOPs stress (Hu et al., 2021a). The developed root system of inoculated plants could make the rhizosphere more oxygenated and created a favorable aerobic condition for the removal of IBU (Zwiener et al., 2002). Besides, the exudates of host plant roots can be enhanced by AMF from quality to quantity, which has been proved to directly promote the development of distinct microbial communities in the rhizosphere and the degradation of EOPs (Hage-Ahmed et al., 2013). Leyval (2006) investigate the contribution of AMF to phenanthrene biodegradation and found that the presence of AMF modified the structure of bacterial populations in the mycorrhizosphere and increased the density of culturable heterotrophic and phenanthrene degrading bacteria beyond the immediate rhizosphere. Therefore, AMF may increase aerobic metabolizing microorganisms in CW systems, thereby significantly increasing the removal of IBU and the concentrations of aerobic metabolites, such as 2-OH IBU, CA IBU, and 4'-OH DCF (**Fig. 4.5 and 4.6**). These results are in accordance with a recent finding reported by Hu et al. (2021a), who found that the presence of AMF created better aerobic conditions in CW systems and increased the aerobic metabolites pathway of IBU by 5.17%. In addition, the external mycelium of AMF might also have the great potential capacity to

absorb EOPs from the rhizosphere, thereby reducing the content of CEC in the aqueous phase. This was supported by Gao et al. (2010). They confirmed that AMF hyphae could take up fluorene and phenanthrene from contaminated soil and transport them to plant roots.

However, the results of our study also indicated that the type of substrate in CWs influenced the effect of AMF on EOPs removal. The effects of AMF on the removal of IBU and DCF were significant in sand systems but limited in adsorptive substrate systems (perlite, vermiculite, and biochar). This may be because adsorptive substrates probably have potential negative impacts on AMF that could limit the performance of AMF on EOPs removal. Abou El Seoud (Abou El, 2021) investigate the effect of biochar addition on AMF performance and found that the decrease of mycorrhizal parameters (AMF colonization, AMF spores, and hyphae length) was significant with an increase in biochar addition. The negative impacts of adsorptive materials on AMF were also verified by another study (Kolton et al., 2017). Besides, the limited effects of AMF in CWs may be attributed to the addition of perlite, vermiculite, and biochar increased water retention (**SI, Table S4.3**), which has been supported by Turner and Friese (1998), who found that the activity of AMF decreased with increased soil moisture. In addition, the positive effect of adsorptive substrates on microbial activity and plant growth might also directly promote the removal of EOPs in CWs by enhancing the cooperation among plant uptake, microbial degradation, and biofilm adsorption (Dordio and Carvalho, 2013). Therefore, the role of CW substrate on the removal of EOPs in CWs might be more predominant than that of AMF. This was supported by the results of two-way ANOVA that the interaction of substrates and AMF (substrates * AMF) showed a more significant effect on the concentrations of IBU and DCF than that of AMF (**SI, Table S4.6**).

EOPs and their metabolites in the rhizosphere and plant tissues

The contents of parent EOPs (IBU and DCF) and their metabolites (2-OH IBU, CA IBU, and 4'-OH DCF) in the rhizosphere and plant tissues (both roots and shoots) were shown in **Table 4.2**. Compared with AMF- treatment systems, the contents of parent EOPs and metabolites (2-OH IBU and CA IBU) in the rhizosphere from AMF treatment systems were increased in sand and perlite but decreased in vermiculite and

biochar systems. Moreover, the contents of IBU in the roots of *G. maxima* from sand, perlite, vermiculite, and biochar systems with AMF addition were significantly increased by 33%, 165%, 136%, and 48%, respectively ($p < 0.05$). Compared with the non-inoculated plant, the contents of DCF in the roots of the inoculated plant were significantly increased in sand and perlite systems but decreased in vermiculite and biochar systems ($p < 0.05$). Meanwhile, the contents of IBU and DCF in the shoots of *G. maxima* in CW systems (except sand systems) with AMF+ treatments were significantly higher than those systems with AMF- treatments.

AMF can influence the accumulation of EOPs in the rhizosphere and the uptake of EOPs by plants (Hu et al., 2021a). The possible reason is that the mycorrhizal pathway contributes to the uptake of EOPs by the host plant. Gao et al. (2010) demonstrated that the extraradical mycelium of AMF could extend into the contaminant-spiked compartment to absorb EOPs (fluorene and phenanthrene), then transported EOPs to the roots of host plants grown in an un-spiked compartment, which significantly increased the contents of EOPs in the roots of inoculated ryegrass (*Lolium multiflorum Lam.*). Meanwhile, Cameron et al. (Cameron et al., 2013) pointed out that with the development of arbuscules in the roots of inoculated plants, AMF can significantly influence the composition and activity of microorganisms in the mycorrhizosphere through altering the chemical composition of root exudates, which might also contribute to the bioaccumulation and biodegradation of EOPs in the rhizosphere. In line with previous studies (Xu et al., 2009), we also found that AMF significantly ($p < 0.05$) decreased the accumulation of parent EOPs (IBU and DCF) and metabolites (2-OH IBU and CA IBU) in the rhizosphere in perlite and biochar systems, promoted the uptake of IBU by the roots of inoculated *G. maxima* in all substrate systems and inhibited the transportation of parent EOPs from plant roots to shoots in sand systems. Conversely, AMF significantly decreased the accumulation of EOPs and metabolites in the rhizosphere in vermiculite and biochar systems and promoted the transportation of parent EOPs from plant roots to shoots in perlite, vermiculite, and biochar systems. This could due to the type of substrate directly affects the growth of wetland plants and the development of microorganisms (including AMF) (Latef et al., 2016), which influenced the role of AMF in the transformation and translocation of EOPs in CWs.

Besides, AMF may play an important role in the distribution of EOPs' metabolites in plant tissue. An explanation may be that AMF can influence gene expression and enzymatic activities in plant cell organelles to enhance the transformation of EOPs in

plant tissues. Previous studies demonstrated that AMF had positive effects on improving the activities of enzymes related to the metabolism of IBU and DCF, including cytochrome P450 monooxygenase (Bellés et al., 2008), glycosyltransferase (Chen et al., 2018), and glutathione-S transferase (García-Sánchez et al., 2014). In our study, however, we found that the contents of 2-OH IBU in the roots of inoculated *G. maxima* were significantly decreased in sand systems but increased in perlite, vermiculite, and biochar systems ($p < 0.05$). In contrast, the contents of 2-OH IBU in the shoots of inoculated *G. maxima* were significantly decreased in sand and perlite systems but increased in vermiculite and biochar systems ($p < 0.05$) (**Table 4.2 and Fig. 4.4**). This indicated that the role of AMF on the metabolism of EOPs in plant tissues might be influenced by CW substrate, which was supported by the results of two-way ANOVA that substrates can significantly affect the metabolism of IBU and DCF in AMF+ treatment systems ($p < 0.01$) (**SI, Table S4.6**). In addition, the accumulation of IBU, DCF, and their metabolites were different in inoculated plant tissues from the same substrate systems (**Table 4.2**). The main reason could be that the differences in physicochemical properties, such as $LogK_{ow}$, functional groups, and chemical structures (**SI, Table S4.1**), could influence their transformation and translocation in inoculated plants.

However, direct evidence that AMF is directly involved in the removal of EOPs in CWs is still lacking. It is not clear that the effect of AMF on the metabolic process of EOPs in different substrate CWs. Consequently, additional studies are necessary to investigate whether the mycorrhizal pathway can play an important role in the phytoremediation of EOPs in CWs. Further attention should be paid to the effects of AMF on the metabolic pathway of EOPs in CWs, such as microbial degradation, absorption by substrate and microbes, and plant uptake, to get a better insight into the functional roles of AMF in EOPs removal.

4.5 Conclusion

AMF colonization can be found in the roots of *G. maxiam* from all substrate systems. Meanwhile, the addition of adsorptive substrates (perlite, vermiculite, and biochar) promoted the formation of AMF symbiosis. AMF enhanced the tolerance of *G. maxima* to the stress of IBU and DCF by increasing antioxidant enzymes (POD and SOD) and soluble contents and decreasing oxidative damage (MDA and $O_2^{\bullet-}$). The addition of adsorptive substrates improved the removal efficiencies of IBU and DCF in CWs because they had positive effects on enhancing the accumulation of EOPs in the rhizosphere and promoting the uptake of EOPs by plant roots. Moreover, AMF promoted the removal of IBU and DCF in sand systems but limited their removal in adsorptive substrate systems. However, the contents of EOPs metabolites (2-OH IBU, CA IBU, and 4'-OH DCF) were decreased in the effluent of AMF+ treatment systems. Furthermore, the role of AMF on the removal of IBU and DCF in CW systems was influenced by CW substrate. The contents of parent EOPs and metabolites in the rhizosphere were increased in sand and perlite systems but decreased in vermiculite and biochar systems by AMF addition. AMF improved the uptake of IBU by the roots of *G. maxima* in all substrate systems and promoted the transformation of IBU and DCF from plant roots to shoots in all substrate systems (except sand systems). Therefore, this study indicated that the application of AMF in CWs has positive effects on the removal of EOPs.

4.6 Supplementary Materials

Methods and materials

- Preparation for the seedlings of wetland plants

Tables:

- Table S4.1. Physical-chemical capacities of EOPs and their metabolites
- Table S4.2. The actual concentration of IBU and DCF in wastewater treatment plants
- Table S4.3. The mean volume of the effluent and influent in different systems
- Table S4.4. *G. maxima* biomass in different substrate systems.
- Table S4.5. Two-way ANOVA analysis of physiological parameters in different substrate systems
- Table S4.6. Two-way ANOVA analysis of EOPs and their metabolites in different substrate CW systems
- Table S4.7. The effect of substrates on the removal of IBU and DCF in AMF- and AMF+ treatment systems

Figures:

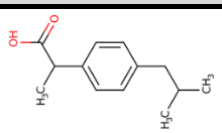
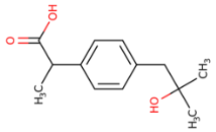
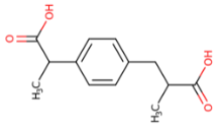
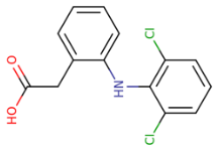
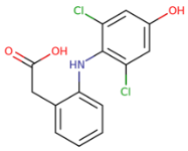
- Fig. S4.1. AMF colonization in different substrate CW systems
- Fig. S4.2. Physiological parameters of plant shoots
- Fig. S4.3. Soluble protein contents of plant tissues in different substrates CW systems
- Fig. S4.4. The mass removal rate of EOPs in different CW systems

Methods and materials:

Preparation for the seedlings of wetland plants (*Glyceria maxima*): The roots of each plant were washed carefully with tap water to remove soil, and then sterilized in 75% ethanol for 10 s, 1% NaClO solution for 10 minutes, finally washed five times with sterile distilled water.

Tables:

Table S4.1. Physical-chemical capacities of EOPs and their metabolites.

EOPs	Structure	Molecular	$LogK_{ow}$	pK_a
Ibuprofen (IBU)		206.30	3.26	4
2-hyroxy ibuprofen (2-OH IBU)		222.28	4.15	4.63
Carboxy ibuprofen (CA IBU)		236.2637	2.78	3.97
Diclofenac (DCF)		296.15	4.26	4
4’-hyroxy diclofenac (4-OH DCF)		312.15	3.96	3.76

$LogK_{ow}$ and pKa (Strongest Acidic) values were collected form ChemAxon (https://chemaxon.com)

Table S4.2. The actual concentration of IBU and DCF in wastewater treatment plants. The concentrations of ibuprofen in the influent and effluent of wastewater treatment plant (WWTP) were 1.7-373.1 μg/L and 0.8-48.2 μg/L, respectively, while the concentrations of diclofenac in the inflow and effluent of WWTP were 0.7-115.1 μg/L and 0.4-2.4 μg/L, respectively.

EOPs	Concentration (μg/L)	Reference
Inflow		
Ibuprofen	13.1	Lindqvist et al. (2005)
	3. 7-19.0	Carballa et al. (2008, 2004)
	93.5 (0-603.0)	Santos et al. (2009)
	8.5	Lishman et al. (2006)
	1.7-33.8	Petrie et al. (2015)
	12.1-373.1	Santos et al. (2007)
	10.0-220.9	Madikizela and Chimuka (2017)
Diclofenac	1.2-3.4	Carballa et al. (2008)
	0.7-1.5	Petrie et al. (2015)
	2.6-115.1	Madikizela and Chimuka (2017)
Outflow		
Ibuprofen	3.1-27.3	Ashton et al. (2004)
	0.8-48.2	Santos et al. (2007)
Diclofenac	0.4-2.4	(Ashton et al., 2004)

Table S4.3. The mean volume of the effluent and influent in different systems.

Systems	Influent (L)	Effluent (L)	
		AMF-	AMF+
Sand	2	1.14	1.12
Perlite	2	0.72	0.68
Vermiculite	2	0.66	0.62
Biochar	2	0.54	0.44

Table S4.4. *G. maxima* biomass in different substrate systems.

Biomass		Sand	Perlite	Vermiculite	Biochar
Root weight	AMF-	125.33±1.53	136.33±15.31	316.33±2.08	149.67±16.26
(g)	AMF+	135.67±9.02	153.67±11.93	354.03±16.37	158.33±10.02
Shoot weight	AMF-	78.33±7.02	109.33±3.51	156.21±6.24	111.67±8.62
(g)	AMF+	85.67±4.73	126.33±6.03	171.15±8.19	124.33±8.02
Root length	AMF-	20.33±2.08	24.33±2.08	39.01±1.04	23.12±3.32
(cm)	AMF+	23.67±1.15	35.67±1.15	45.11±1.09	34.67±2.08
Shoot height	AMF-	33.67±2.08	43.18±3.61	61.33±1.53	54.67±3.21
(cm)	AMF+	40.33±1.53	58.33±3.51	71.33±0.58	58.03±3.12

Table S4.5. Two-way ANOVA analysis of physiological parameters in different substrate CW systems with AMF- and AMF+ treatments. Substrates and AMF treatments as main factors and substrates * AMF treatments as interaction effect. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. MDA: malondialdehyde; $O_2^{\bullet-}$: superoxide anion; POD: peroxidase; SOD: superoxide dismutase.

Physiological parameters	Substrates	AMF	Substrates*AMF
Roots			
MDA	***	***	0.0617
$O_2^{\bullet-}$	***	***	***
POD	***	***	0.07635
SOD	***	***	0.295
Protein	***	***	*
Shoots			
MDA	***	***	0.649
$O_2^{\bullet-}$	***	***	***
POD	***	***	0.875
SOD	***	***	0.519
Protein	***	***	*

Table S4.6. Two-way ANOVA analysis of EOPs and their metabolites in different substrate CW systems under AMF- and AMF+ treatments. Substrates and AMF treatments as main factors and substrates * AMF treatments as interaction effect. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. -: no data.

CW systems	EOPs and their metabolites	Substrates	AMF	Substrates*AMF
Effluent	Ibuprofen	***	0.705	***
	2-Hydroxy Ibuprofen	***	***	***
	Carboxy Ibuprofen	***	***	***
	Diclofenac	***	*	***
	4'-Hydroxy Diclofenac	***	0.145	0.102
Rhizospheres soil	Ibuprofen	***	***	***
	2-Hydroxy Ibuprofen	-	-	-
	Carboxy Ibuprofen	-	-	-
	Diclofenac	-	-	-

CW systems	EOPs and their metabolites	Substrates	AMF	Substrates*AMF
	4'-Hydroxy Diclofenac	-	-	-
Roots	Ibuprofen	***	***	***
	2-Hydroxy Ibuprofen	***	***	***
	Carboxy Ibuprofen	***	-	-
	Diclofenac	***	***	***
	4'-Hydroxy Diclofenac	***	-	-
Shoots	Ibuprofen	**	**	**
	2-Hydroxy Ibuprofen	***	***	***
	Carboxy Ibuprofen	-	-	-
	Diclofenac	***	***	***
	4'-Hydroxy Diclofenac	-	-	-

Table S4.7. The differences in the removal of ibuprofen and diclofenac among the four substrate systems. *: $p < 0.05$; **: $p < 0.01$.

Significance of substrate	Ibuprofen		Diclofenac	
	AMF-	AMF+	AMF-	AMF+
Sand-Biochar	**	**	**	**
Sand-Perlite	**	**	**	**
Perlite-Biochar	**	**	**	**
Vermiculite-Biochar	**	**	**	**
Vermiculite-Perlite	0.642	0.735	0.999	**
Vermiculite-Sand	**	**	**	**

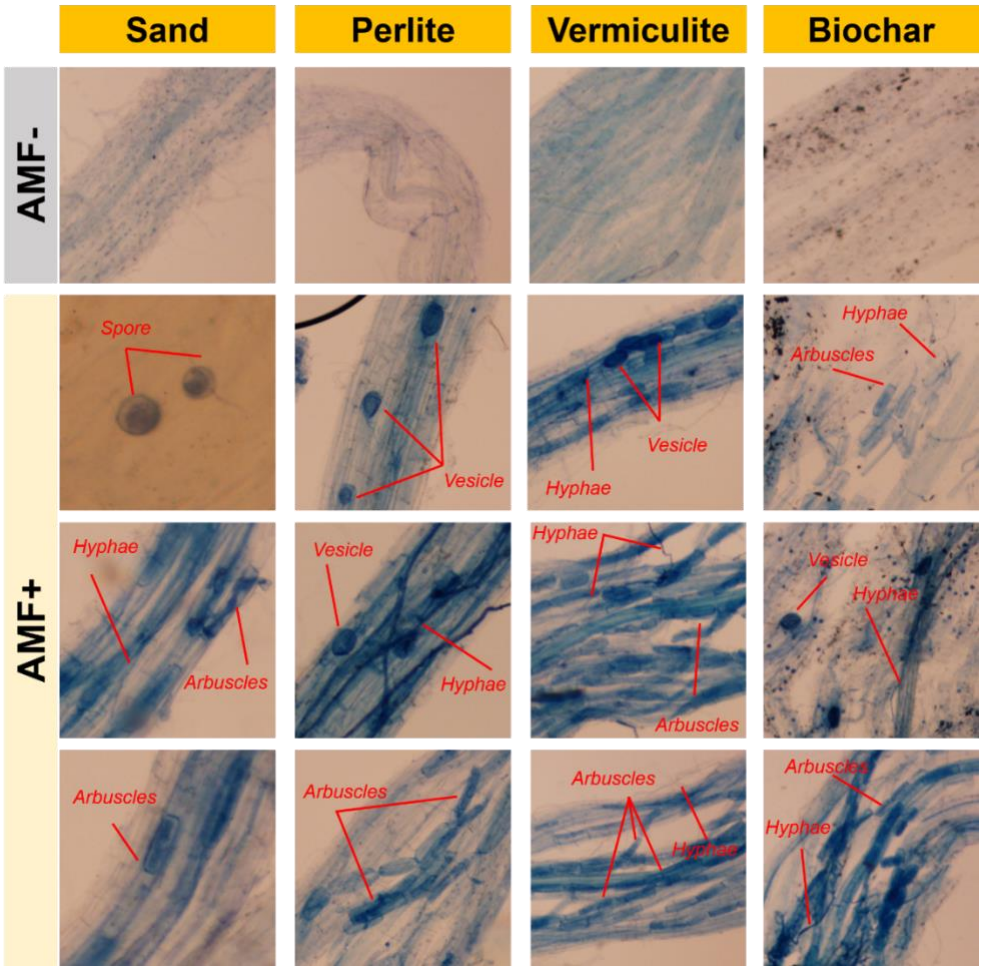


Fig. S4.1. The observations of AMF colonization in the roots of *G. maxima* from different substrate systems.

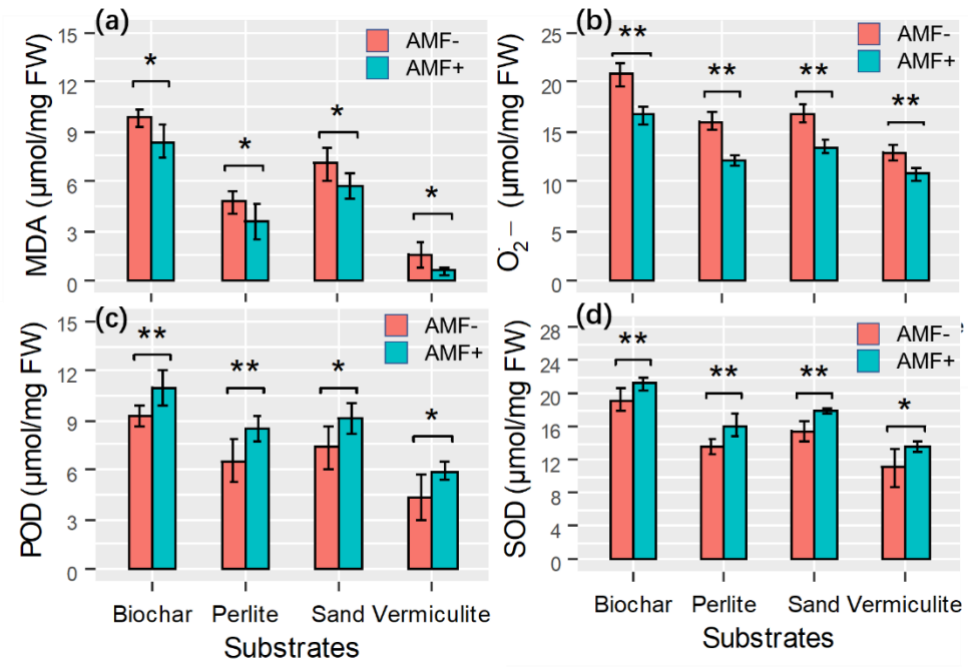


Fig. S4.2. Physiological parameters of plant shoots: (a) MDA, (b) $O_2^{\bullet-}$, (c) POD, (d) SOD. The data are the means ± standard errors (n=3). * and ** show the significant difference ($p < 0.05$) and extremely significant difference ($p < 0.01$), respectively, in AMF- and AMF+ treatments. MDA: malondialdehyde; $O_2^{\bullet-}$: superoxide anion; POD: peroxidase; SOD: superoxide dismutase.

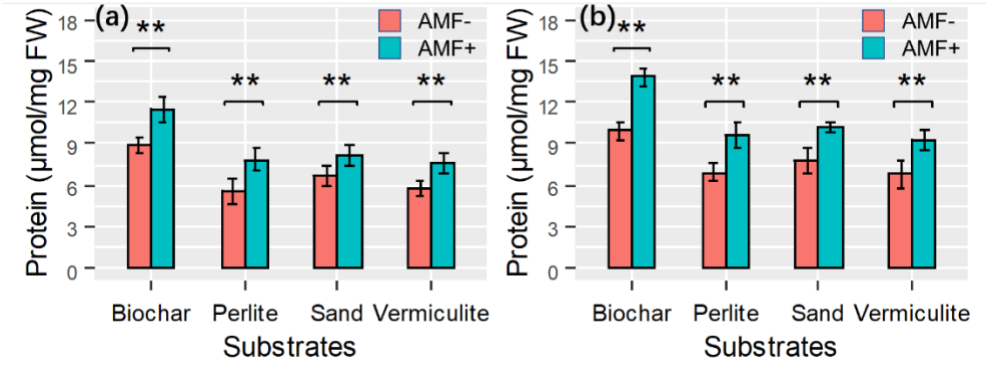


Fig. S4.3. Soluble protein contents of plant tissues in different substrates CW systems: (a) Roots, (b) Shoots. The data are the means ± standard errors (n=3). ** shows the extremely significant difference ($p < 0.01$) in AMF- and AMF+ treatments.

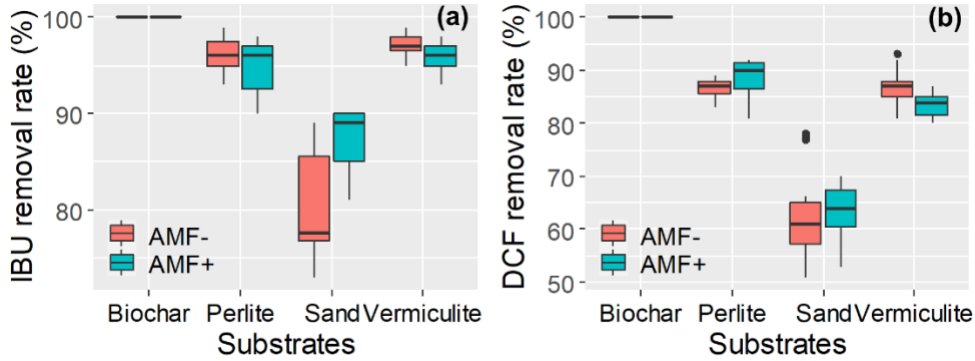


Fig. S4.4. The mass removal rate of EOPs in different CW systems. (a) ibuprofen (IBU) and (b) diclofenac (DCF). The data are the means ± standard errors (n=15).

Chapter V

Application of arbuscular mycorrhizal fungi for pharmaceuticals and personal care productions removal in constructed wetlands with different substrate

Bo Hu, Shanshan Hu, Jan Vymazal, Zhongbing Chen

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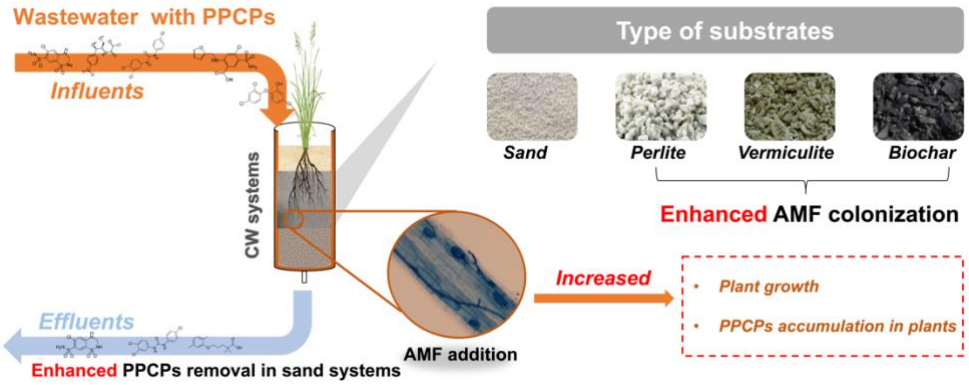
Contents

5.1 Abstract

This study investigated the effects of substrates (sand, perlite, vermiculite, and biochar) on the colonization of arbuscular mycorrhizal fungi (AMF) in the roots of *Glyceria maxima* in constructed wetlands (CWs) and the impacts of AMF inoculation on the removal of six selected pharmaceuticals and personal care products (PPCPs). Results showed that the application of adsorptive substrates (perlite, vermiculite, and biochar) in CWs had positive effects on AMF colonization. AMF could influence the uptake and translocation of PPCPs in plant tissues. The amount of PPCPs in the roots of inoculated plants was increased by 21-193% and 67-196% in sand and vermiculite systems but decreased by 13-55% and 51-100% in perlite and biochar systems, respectively, compared to the non-inoculated controls. Meanwhile, AMF enhanced the translocation of PPCPs to plant shoots, resulting in higher accumulations of PPCPs in the shoots of inoculated plants than that of non-inoculated plants. AMF had positive effects on removing PPCPs in sand systems but insignificant effects in adsorptive substrate systems. Therefore, these results indicated that the symbiotic relationship between AMF and plant roots could affect the accumulation and translocation of PPCPs in plants, and substrate type can influence this function. This study could be a starting point for exploring the potential role of AMF in PPCPs removal in CWs.

Graphical abstract:



5.2 Introduction

In recent decades, the widespread presence of pharmaceuticals and personal care products (PPCPs) in natural water has attracted more and more attention (Dos Santos et al., 2021; Ilyas and Van Hullebusch, 2020). It has been documented that PPCPs can be continuously discharged into the surface, ground, and coastal water bodies via various sources, such as hospitals, industrials, livestock farms, wastewater treatment plants (WWTPs), etc. (Chen et al., 2016b). Consequently, PPCPs, as pseudo-persistent pollutants, can be frequently detected in the aquatic environment with nonnegligible concentrations (Rathi et al., 2021). Previous studies indicated that PPCPs have potential toxicological effects on ecosystems and human beings through bioaccumulation and biomagnification in food chains (Fent et al., 2006; Rathi et al., 2021). For these reasons, the removal of PPCPs from the wastewater is critically needed to protect aquatic ecosystems and human health. Hitherto, numerous wastewater treatment technologies have been developed for removing PPCPs, such as ozonation, Fenton oxidation, ionizing irradiation, membrane bioreactor, and other combined chemical and biological treatments (Wang and Wang, 2016). However, these technologies are not entirely feasible for widespread application for small communities because of high energy input and high-cost requirements. Thus, there are growing appeals for developing more cost-effective treatment strategies to furtherly reduce the risk of PPCPs into the ecosystems.

Constructed wetland (CW) is an eco-friendly and cost-effective wastewater treatment technology that has been proven to be efficient in removing PPCPs (Vymazal et al., 2017). PPCPs in CW systems can be removed in various processes, including plant uptake, adsorption, microbial degradation, hydrolysis, and volatilization (Ilyas and Van Hullebusch, 2020). However, some PPCPs in CWs may also cause adverse effects on the stability of the system operation. For example, a previous study indicated that triclosan could influence the growth of wetland plants (e.g., seed germination and root development) even at a low concentration (0.6 μg/L) (Stevens et al., 2009). Meanwhile, some PPCPs might have chronic toxicity for animals (e.g., *Daphnia*), microalgae, and microorganisms (e.g., bacteria) in CWs (Breitholtz et al., 2012; Crane et al., 2006; Rathi et al., 2021). Therefore, it is essential to reduce or eliminate the negative impact of PPCPs on the stability of wetland systems, which is beneficial for the removal of PPCPs in CWs.

Arbuscular mycorrhizal fungi (AMF) are an essential component of soil microorganisms globally, forming a mutualistic symbiosis with more than 80% of terrestrial plants (Smith and Read, 2008). It is well known that AMF could play significant roles in enhancing the resistance against biotic and abiotic stresses, such as heavy metals (Hu et al., 2021b), drought (Ren et al., 2019), and organic contaminants (Gao et al., 2010). A review conducted by Wang et al. (2020) suggesting that the benefits and mechanisms of AMF in ameliorating organic contaminant residues in crops can be summarized as follows, such as promoting nutrient uptake and water acquisition, alleviating oxidative stress of the host plant, enhancing activities of contaminant degradation-related enzymes, changes in soil structure and contaminant-relating microorganisms, influencing the bioavailability of contaminants, and the accumulation and sequestration of contaminants by AMF structures. Similarly, experimental evidence has been obtained by Corgié et al. (2006) and Gao et al. (2010), who found that AMF can promote the removal of polycyclic aromatic hydrocarbons (PAHs, e.g., fluorene and phenanthrene) by enhancing the uptake of the terrestrial plant (*Lolium multiflorum* Lam.) and modifying the structure and density of bacterial populations in the mycorrhizosphere. However, the occurrence of AMF in wetland systems is generally regarded as lower than that in terrestrial environments according to the natural preference of AMF for aerobic conditions. Nevertheless, there is growing evidence that AMF is widespread in wetland habitats, such as mangroves (Wang et al., 2015), marshes (Zhang et al., 2014b), constructed and natural wetlands (Fester, 2013; Wang et al., 2018a) in the last decades. The symbiotic relationship between AMF and plant roots has been found in 99 families of wetland plants (Xu et al., 2016). Hence, the application of AMF in wetland ecosystems has recently received research interest (Barbera et al., 2020; Hu et al., 2021b). Our previous studies also have proven that AMF symbiosis could contribute to plant growth (*Glyceria maxima*) and pharmaceuticals removal (ibuprofen and diclofenac) in CWs filled with sand (Hu et al., 2021a). However, there is little knowledge about the effect of AMF on PPCPs in CWs with different substrates.

Substrates, also known as support material/matrix, media and filling material, are one of the critical components in CWs, which play significant roles in CWs, such as providing a carrier for plant growth and biofilm development and removing pollutants through absorption and adsorption (Wu et al., 2015b). Based on the material of substrates, they can be divided into two categories: conventional substrates (e.g., sand, soil, gravel) and emerging substrates (e.g., construction wastes, tire chips, zeolite, perlite, vermiculite, biochar, etc.) (Yang et al., 2018). Previous studies have well

demonstrated that the selection of substrates directly influences PPCPs removal (Li et al., 2014) and affects the composition of the microbial community in CWs (Bai et al., 2021). This indicated that the type of substrate might also affect the establishment of symbiosis between AMF and plant roots. However, there is a significant knowledge gap regarding whether the functional role of AMF in PPCPs removal can be affected by CW substrates.

Based on the above, this study aims to fill the research gap by investigating the effects of AMF on PPCPs removal in CWs with different substrates. Therefore, the specific objectives of this study were: 1) to explore the effects of AMF on PPCPs removal in CWs; 2) to evaluate the impacts of AMF on the translocation and transformation of PPCPs in CW filled with different substrates. Four substrates, including sand, perlite, vermiculite, and biochar, were selected covering the two main categories of substrate. The selection of substrate in this study is mainly because they can perform markedly different in terms of PPCPs removal and the development of microorganisms due to their various properties (Bai et al., 2021; Yang et al., 2018). To the best of our knowledge, the present study is the first time to explore the functional role of AMF in PPCPs removal in CWs filled with different substrates. The findings of this study attempted to provide new insights into the application of AMF for PPCPs removal in real-scale wetland systems.

5.3 Materials and methods

5.3.1 Experimental materials

Hydrochlorothiazide, chloramphenicol, furosemide, gemfibrozil, triclocarban, and triclosan were selected as the target PPCPs in this study, mainly because they are modern current-use chemicals and have attracted increasing concern due to their persistence in the environment (**Supporting Information (SI), Table S5.1**) and potential for deleterious effects (Gavrilescu et al., 2015). In recent decades, they have been detected in various environmental samples, including the influent and effluent of WWTPs, sludges, sediments, and rivers (**SI, Table S5.2**). PPCPs with a purity > 98% were provided by Sigma-Aldrich (Schnelldorf, Germany). The physicochemical properties of PPCPs are shown in SI, **Table S5.3**.

Glyceria maxima is a common wetland species in Europe, selected as the wetland plant in this study. The seedlings of *G. maxima* (average height of 15 cm) were obtained from a local pond at the Czech University of Life Sciences Prague, Czech Republic. Before transplantation, plant roots were surface sterilized with 75% ethanol (about 10 s), followed by 1% NaClO (about 10 min), finally washed with distilled water (5 times).

Rhizophagus irregularis (BEG140) was the fungal inoculum because it can be frequently observed in wetland ecosystems, including *G. maxima* (Xu et al., 2016). The detailed information of the fungal inoculum was shown in our previous study (Hu et al., 2021a).

Siliceous sand (0.1-0.6 mm), expanded perlite (0.1-2.0 mm), expanded vermiculite (0.7-2.0 mm), and biochar (0.3-6.0 mm) were selected as substrates in this study. Sand is a conventional substrate in CWs, while perlite, vermiculite, and biochar are the emerging substrates employed in CWs in recent years (Yang et al., 2018). In general, based on the adsorption capacity of PPCPs, the selected substrates can be classified into low/no sorption (sand), moderate sorption (perlite and vermiculite), and high sorption (biochar). The detailed information of the selected substrates was shown in SI, and their physicochemical property was shown in **Fig. 5.1**.

5.3.2 Experimental setup

Experiments were performed in PVC-U materials columns (15×55 cm, diameter × height), as shown in **SI, Fig. S5.1**. Each column was divided into three layers: 15 cm gravel layer at the bottom; 20 cm substrate layer in the middle; 15 cm sand layer on the top. The middle layers were different among the four substrate systems, filled with sand, perlite, vermiculite, or biochar, respectively. Each substrate system was set two treatments: a) AMF+ treatments: 350g fungal inoculum was added and mixed with the middle layer substrate; b) AMF- treatments: 350g sterilized fungal inoculum mixed with 100 mL inoculum filtrate then added into the middle layer substrate (**SI, Fig. S5.1b**). Each treatment had three replicates. All substrates were sterilized at 120 ℃ for three hours before their addition.

Two ramets of *G. maxima* were transplanted into the middle layer of each column. Since the occurrence of AMF shows a negative correlation with water depth and duration of flooding (Hu et al., 2020b), CWs were operated with a tidal flow condition

(2 L per 4 d) during the whole experiment, in which the flood-and-drain cycle was set to rhythmically occur every 4 d, providing 2 h "flood" phase and 94 h "drain" phase. In the first two months (from June to August), CWs were fed with 10% strength synthetic wastewater. Then six selected PPCPs (50 μg/L of each PPCPs) and 100% strength synthetic wastewater were mixed and added to CWs until the end of this study (from September to November). The added concentration of each PPCPs was related to their detected concentrations in the environment (**SI, Table S5.2**). The detailed composition of synthetic wastewater is shown in our previous study (Hu et al., 2021a). This experiment was conducted under a transparent plastic roof for protection against rain but ensuring outdoor environment conditions. The temperature changes during the experiment were ranged from -5℃ to 30℃, as shown in **SI, Fig. S5.4**.

5.3.3 Sample analysis

Plant sampling and measurement

Before adding those PPCPs, two ramets of *G. maxima* were harvested to measure AMF colonization in plant roots. The measurement methods are shown in our previous study (Hu et al., 2021a). *G. maxima* were harvested at the end of this experiment, then divided into underground (root) and aboveground (shoot) parts. Shoot height and root length were directly measured by fresh samples, while shoot and root weight were measured after oven drying at 40 ℃ for 120 h.

PPCPs in CWs

After adding PPCPs, their concentrations in the influent and effluent from each CWs were detected every 12 days. At the end of the experiment, the content of PPCPs in plant tissues and the rhizosphere soil was measured. The methods for PPCPs measurement is shown in our previous study (Hu et al., 2021a).

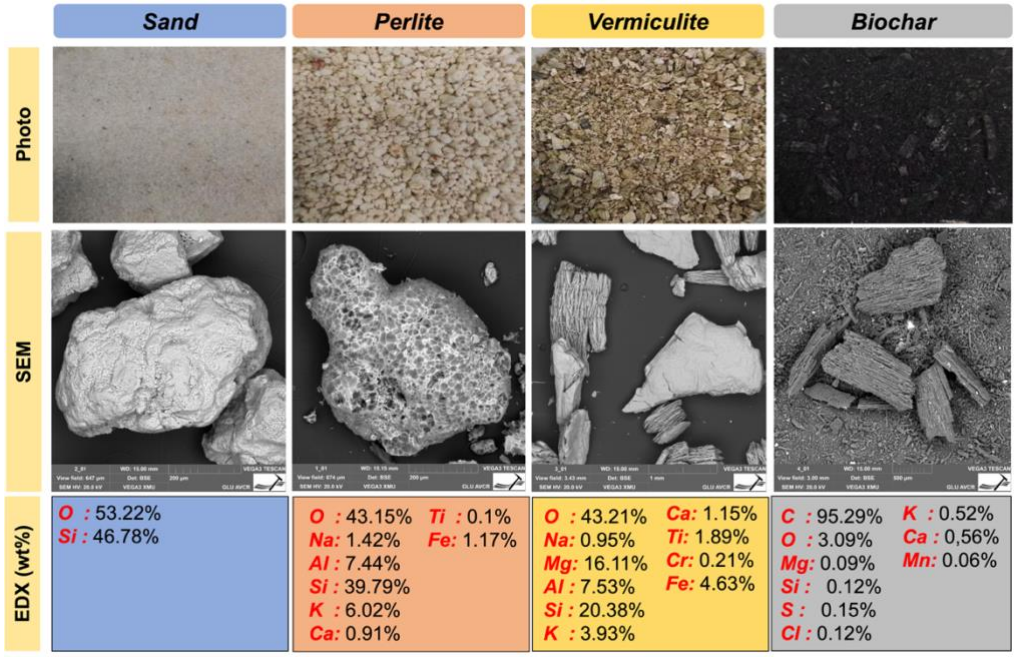


Fig. 5.1 The characteristics of substrates. SEM images and EDX spectra of sand, vermiculite, perlite, and biochar.

Operating condition and water quality

Water samples were collected from influent and effluent every eight days to measure operation condition and water quality of CWs. The operating conditions of CWs, including dissolved oxygen (DO), pH, oxidation-reduction potential (ORP), and electrical conductance (EC), were measured by a multi-parameter portable meter (Multi 3630 IDS, WTW). The conventional wastewater parameters, such as total organic carbon (TOC), total nitrogen (TN), PO_4^{3-}-P, NO_3^--N, NO_2^--N, and NH_4^+-N, were also measured according to the previous study (Hu et al., 2021a).

5.3.4 Data analysis

PPCPs removal in different CW systems was compared by their mass removal efficiency calculated by mass balance (Hu et al., 2021a). Statistical analysis and data

visualization were achieved by R software (Version 4.0.1). The results were analyzed through the student's t-test and a two-way analysis of variance (ANOVA). Differences were considered significant when $p < 0.05$. Principle component analysis (PCA) and cluster analysis were carried out to examine the impact of AMF and substrates on plant growth, operating parameters of CWs, and PPCPs concentrations in wastewater.

5.4 Results and discussion

5.4.1 AMF colonization

The symbiosis between AMF and *G. maxima* can be found in all substrate CW systems under AMF treatments (**SI, Fig. S5.2**). However, mycorrhizal status in plant roots showed differences among the four substrate CWs (**SI, Fig. S5.3**). The intensity of mycorrhizal inoculation in sand (10.6%) systems was significantly lower than that in perlite (26.2%), vermiculite (58.2%), and biochar (24.4%) systems ($p < 0.05$). Similarly, the frequency of mycorrhiza and the arbuscule abundance in sand systems were lower than in perlite, vermiculite, and biochar systems.

In our study, AMF colonization in sand systems was lower than that in adsorptive substrate systems. A possible explanation is that the porous structures of adsorptive substrates in CWs can be considered as a perfect bio-carrier for hyphae (Lehmann and Joseph, 2012; Wen et al., 2016), as shown in **Fig. 5.1**, which could provide a more favorable environment for the establishment of mycorrhizal symbiosis. Meanwhile, the difference in oxygen conditions may affect mycorrhizal symbiosis in different substrate systems. The dissolved oxygen contents in sand systems (1.8-3.0 mg/L) were significantly lower than that in perlite (5.9-6.3 mg/L), vermiculite (7.0-7.5 mg/L), and biochar (5.1-5.5 mg/L) systems (**Fig. 5.3a**), indicating that AMF colonization increased with the increase in the dissolved oxygen contents. Zhang et al. (2014b) investigated the relationship between rhizosphere oxygen concentration and AMF colonization in the roots of *P. australis* and found that AMF colonization was positively related to oxygen concentration. Therefore, the establishment of symbiosis between AMF and plant roots can be affected by substrates in CWs. The selection of suitable substrates could contribute to the development of AMF, especially the adsorptive materials.

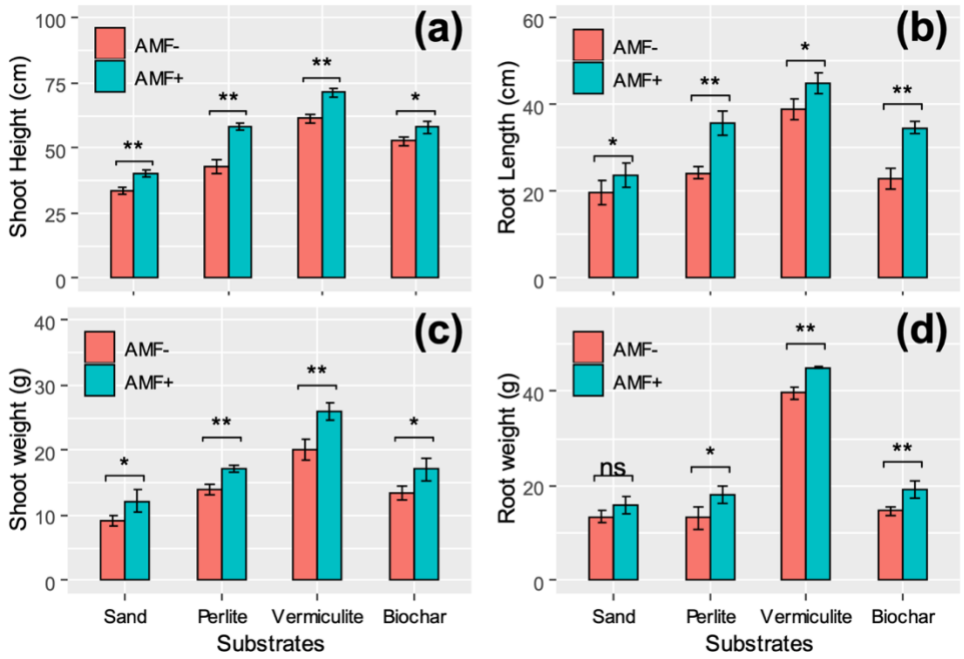


Fig. 5.2. The growth of *G. maxima* in different substrate CW systems. (a) Shoot height; (b) Root length; (c) Shoot weight; (d) Root weight. The data are the means ± standard errors. *: $p < 0.05$; **: $p < 0.01$.

5.4.2 Effects of substrate and AMF on plant growth

Shoot height and root length in sand systems were 19.7-27.7%, 82.2-91.8%, and 13.1-56.4% lower than that in perlite, vermiculite, and biochar, respectively (**Fig. 5.2, a and b**). Similarly, shoot and root weight in sand systems was lower than in other substrate systems (**Fig. 5.2, c and d**). The results of two-way ANOVA indicated that substrate significantly influenced the growth of *G. maxima* in CWs ($p < 0.01$) (**SI, Table S5.4**). These results suggested that the application of adsorptive substrate in CWs could enhance plant growth. A similar conclusion was reached by Chen et al. (2021). They found that biochar can markedly improve the growth of *Iris pseudoacorus* in CWs, resulting in more biomass of both aboveground and belowground parts than that in sand systems. Meanwhile, the positive effects of perlite, vermiculite, and biochar on improving plant growth could be partly due to that they have higher adsorption capacity and cation exchange capacity than sand (Dordio et al., 2007; Yang et al., 2018), thus

increasing the retention of nutrients (e.g., C, N, P, and microelements) and water inside CWs to meet the need of plant growth (Deng et al., 2021). Besides, adsorptive substrates might also act as additional nutrient sources to support plant growth by releasing the essential elements (e.g., C, K, and Mg, as shown in **Fig. 5.1**).

In addition, AMF colonization showed positive effects on plant growth (**SI, Table S5.4**). As shown in **Fig. 5.2**, shoot height, root length, shoot weight, and root weight of AMF inoculated *G. maxima* in the four different substrate systems were 10.1-35.7%, 15.4-51.0%, 22.1-29.5%, and 13.1-34.0% higher than those of the non-inoculated controls, respectively. Smith and Read (2008) suggested that the positive effects of AMF on promoting plant growth attribute to increased uptake of nutrients, especially P. This is mainly due to the extraradical mycelium of AMF absorb nutrients far beyond the area of the rhizosphere to promote the growth of the host plants (Jansa et al., 2019). This is directly in line with a previous study that AMF colonization significantly enhanced the biomass of *P. australis* by promoting N uptake (Liang et al., 2019). Besides, the interactions between AMF and substrates also significantly affected plant growth ($p < 0.01$) (**SI, Table S5.4**), suggesting that the direct impacts of substrate on AMF might also indirectly influence the growth of host plants.

5.4.3 PPCPs in the rhizosphere soil

PPCPs accumulation in the rhizosphere soil of different substrate systems

The concentrations of PPCPs in the rhizosphere soil of the four different substrate systems without AMF addition are shown in **Table 5.1**. Notably, in sand and perlite systems, only gemfibrozil (1 mg/kg and 4 mg/kg) and triclocarban (28 mg/kg and 46 mg/kg) can be found in the rhizosphere soil, while the other PPCPs were not detected. Meanwhile, most PPCPs were observed in the rhizosphere soil of vermiculite and biochar systems except furosemide and triclosan, which the concentrations of hydrochlorothiazide, chloramphenicol, gemfibrozil, and triclocarban were 8-11 mg/kg, 4-5 mg/kg, 18-19 mg/kg, and 13-21 mg/kg, respectively. In addition, the concentrations of hydrochlorothiazide, chloramphenicol, gemfibrozil, and triclocarban in the rhizosphere soil of sand systems were lower than those of vermiculite and biochar

systems. By contrast, the concentration of triclocarban in the rhizosphere of sand (28 mg/kg) and perlite (46 mg/kg) systems was higher than that of vermiculite (21 mg/kg) and biochar (13 mg/kg).

The accumulation of PPCPs in the rhizosphere soil of the four substrate systems showed differences, mainly because substrates have different adsorption capacities for PPCPs (Dordio and Carvalho, 2013; Yang et al., 2018). The higher accumulation of PPCPs in the rhizosphere soil was observed in vermiculite and biochar systems. The possible reason is that vermiculite and biochar's high cation exchange capacity attracts and retains more PPCPs from the aqueous phase of CWs (Yang et al., 2018). Meanwhile, the effects of substrates on plant growth (**Fig. 5.2**) and operating conditions (**Fig. 5.3**) might also influence the development of microorganisms within the CWs (Zhang et al., 2018), and thus leading to differences in the accumulation of PPCPs in the rhizosphere soil of the four substrate systems. As shown in **Fig. 5.1**, vermiculite and biochar have a wider surface area and more pores that can offer potential sites for the growth of microbes to create diverse microbial communities in CWs, which could enhance the bioaccumulation and biodegradation of PPCPs in the rhizosphere soil. Moreover, the biodegradability of PPCPs might also influence their accumulation in the rhizosphere soil. For example, triclocarban shows a higher biological half-life in the environment than other selected PPCPs (**SI, Table S5.1**), indicating that triclocarban has lower biodegradability and is more persistent than others. This could explain why the content of triclocarban in the rhizosphere of sand and perlite systems was higher than that of vermiculite and biochar systems. Overall, the type of substrate and biodegradability of PPCPs could influence the accumulation of PPCPs in the rhizosphere soil of CWs.

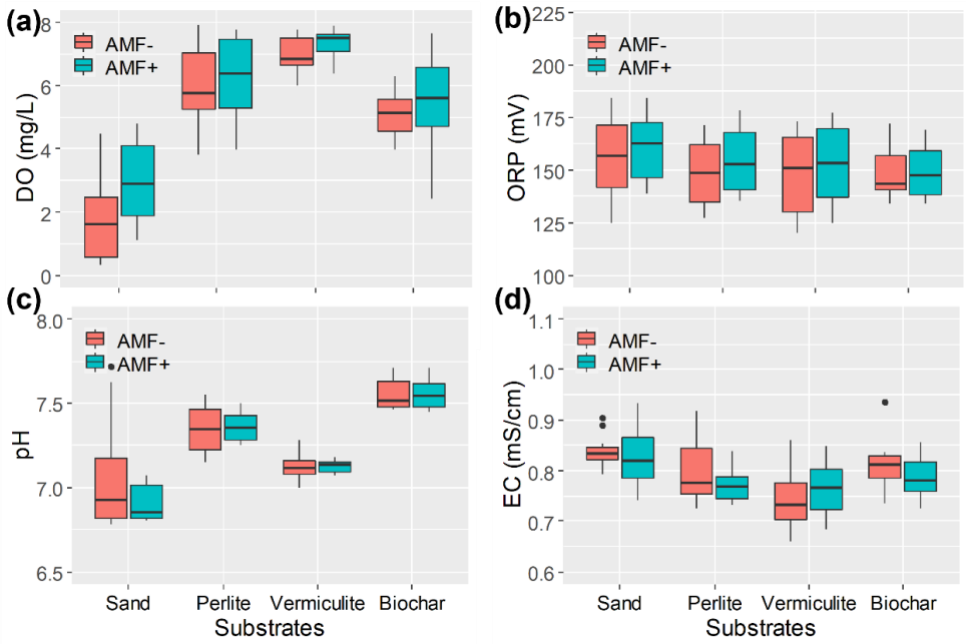


Fig. 5.3. Water quality parameters of the effluent from different substrate systems. (a) DO; (b) ORP; (c) pH; (d) EC. The temporal changes of operating conditions in different systems are shown in SI, Fig. S8.

Effect of AMF on the accumulation of PPCPs in the rhizosphere soil

The contents of all selected PPCPs (except triclosan, which the concentration was below the detection limit) in the rhizosphere soil of sand and perlite systems with AMF addition were higher than those in non-AMF controls (**Table 5.1**). Similarly, the concentrations of chloramphenicol and gemfibrozil in the rhizosphere soil of vermiculite and biochar systems with AMF addition were 40-100% and 184-439% significantly higher than those without AMF addition, respectively ($p < 0.01$). These results were broadly in line with our previous study that the presence of AMF enhanced the accumulations of ibuprofen and diclofenac as well as metabolites (2-hydroxy ibuprofen and carboxy ibuprofen) in the rhizosphere soil of CWs (Hu et al., 2021a). A possible explanation is that AMF promotes the development of microorganisms in the

rhizosphere (both quantitatively and qualitatively) through enhancing the release of exudates (e.g., carbohydrates, flavonoids, amino acids, protein, and other biomolecules) (Monther and Kamaruzaman, 2012), thereby enhancing the adsorption capacity of microbes and biofilm to PPCPs in the rhizosphere soil.

Moreover, AMF also showed the potential to decrease the accumulation of PPCPs in the rhizosphere soil. As shown in **Table 5.1**, a lower hydrochlorothiazide and triclocarban accumulation was observed in the rhizosphere of vermiculite and biochar systems with AMF treatments than that in non-AMF controls. A possible explanation is that AMF enhanced the plant uptake to PPCPs (**Fig. 5.4 and 5.5**). Many studies presented similar results, suggesting that AMF colonization could improve the uptake of organic pollutants (e.g., DDT, atrazine, phenanthrene, and fluorene) by plant roots (Gao et al., 2010; Huang et al., 2007; Wu et al., 2008). Moreover, AMF promoted plant growth (**Fig. 5.2**) and increased DO levels of CWs (**Fig. 5.3a**), which may contribute to enhance the activity of microbes and plant-microbe interactions in the rhizosphere (Wu et al., 2008), thus enhancing the biodegradation of PPCPs in the rhizosphere. This could be another reason that the accumulation of PPCPs in the rhizosphere soil was lower in AMF+ systems. A similar conclusion was reached by Wu et al. (2008), who found that AMF colonization led to an increase in the accumulation of DDT in the roots of alfalfa and significantly increased microbial populations and dehydrogenase activity in the rhizosphere soil. Overall, AMF has the potential to influence the accumulation of PPCPs in the rhizosphere soil of CWs.

Table 5.1. The contents of PPCPs in the rhizosphere soil from different substrate CW systems. Unit: μg/kg dry substrate matter; n.d.: not detected, PPCPs contents were below the detection limited; a and b show significant differences between AMF- and AMF+ treatments in the same substrate systems.

PPCPs	Treatments	Sand	Perlite	Vermiculite	Biochar
Hydrochlorothiazide	AMF-	n.d.	n.d.	11±3.7	8±0.9
	AMF+	8±0.7	13±0.4	n.d.	n.d.
Chloramphenicol	AMF-	n.d.	n.d.	$5±0.2^{a}$	$4±0.2^{a}$
	AMF+	8±0.3	5±0.4	$7±0.5^{b}$	$8±1.1^{b}$
Furosemide	AMF-	n.d.	n.d.	n.d.	n.d.
	AMF+	14±4.4	17±5.9	n.d.	n.d.
Gemfibrozil	AMF-	$1±0.1^{a}$	$4±1.1^{a}$	$19±6.5^{a}$	$18±2.0^{a}$
	AMF+	$31±5.0^{b}$	$34±6.2^{b}$	$54±13.7^{b}$	$97±11.9^{b}$
Triclosan	AMF-	n.d.	n.d.	n.d.	n.d.
	AMF+	n.d.	n.d.	n.d.	n.d.
Triclocarban	AMF-	$28±7.6^{a}$	$46±12.9^{a}$	21±6.9	13±0.8
	AMF+	$41±14.4^{b}$	$78±8.6^{b}$	n.d.	n.d.

5.4.4 PPCPs in plant tissues

Effect of substrates on the accumulation of PPCPs in plant tissues

All the selected PPCPs can be detected in both roots and shoots of non-inoculated *G. maxima* from the four different substrate systems (**Fig. 5.4 and 5.5**). Hydrochlorothiazide, chloramphenicol, furosemide, and gemfibrozil were mainly accumulated in the roots of non-inoculated *G. maxima*. At the same time, the amount of triclosan and triclocarban in the shoots were 1.4-2.5 and 5-7.9 times higher than that in the roots of non-inoculated *G. maxima*, respectively. This suggested that the accumulation and translocation of PPCPs in plant tissues showed differences among compounds. Meanwhile, the results of two-way ANOVA indicated that substrates significantly affected the accumulation of PPCPs in plant tissues ($p < 0.001$) (**SI, Table S5.4**). The highest amount of PPCPs was observed in both roots and shoots of non-inoculated plants from vermiculite systems.

The accumulation of PPCPs in plant tissues has been considered one of the main ways to eliminate PPCPs from the aqueous phase in CW systems (Miller et al., 2016). It has been demonstrated that the uptake and translocation of PPCPs within plant tissues are driven by diffusion, mainly because there are no specific transporters for the movement of PPCPs across the cell membranes of plant cells (Dhir, 2019). Previous studies indicated that the diffusion process of PPCPs into plant tissue depends on the compounds' physicochemical characteristics, such as the dissociation constant pKa, octanol-water partition coefficient K_{ow}, and water solubility (Dordio and Carvalho, 2013; Miller et al., 2016). Meanwhile, the translocation of PPCPs in plants is also related to their physicochemical characteristics (Dhir, 2019). As shown in SI, **Table S5.3**, these properties of the selected PPCPs are different, which could be one of the main reasons that the accumulation of PPCPs in plant roots showed differences among different chemicals (**Fig. 5.4**). For example, the higher accumulation of triclosan and triclocarban in plant tissues was probably related to their higher lipophilicity, which can cause more rapid diffusion across lipid bilayers of the plant cell membrane and thus concentrated in plant tissues (Dhir, 2019; Dordio and Carvalho, 2013). Moreover, the metabolism processes of PPCPs in plants may also influence their accumulation (Miller et al., 2016). Recent evidence has demonstrated that the phytodegradation of PPCPs

(diclofenac and ibuprofen) indeed occurred inside the tissues of aquatic macrophytes (*Typha latifolia* and *P. australis*) by transforming several metabolites (Bartha et al., 2014; He et al., 2017). Triclosan and triclocarban are more persistent in the environment than other selected PPCPs (**SI, Table S5.1**), indicating that they have a low bioavailability, which may also affect their phytodegradation and results in a higher accumulation in plant tissues.

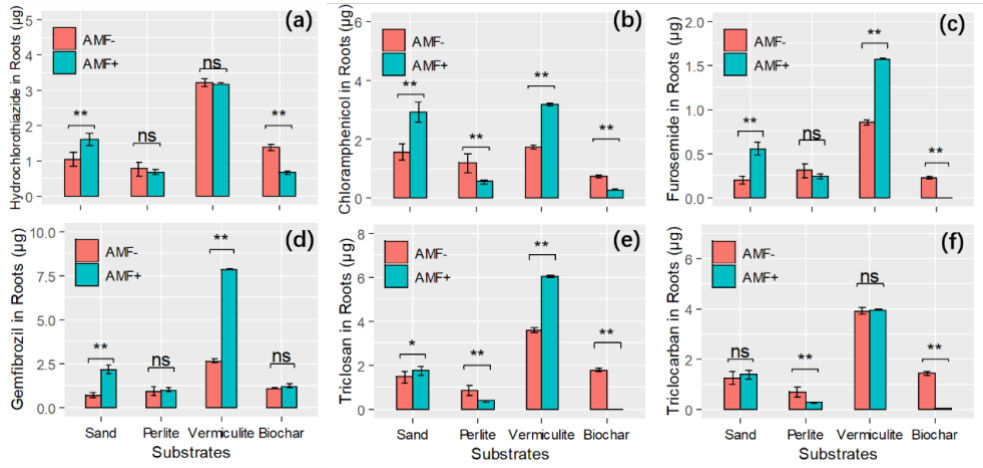


Fig. 5.4. The mass of PPCPs in plant roots (µg). (a) hydrochlorothiazide; (b) chloramphenicol; (c) furosemide; (d) gemfibrozil; (e) triclosan; (f) triclocarban; The data are the means ± standard errors. ns: no differences; *: $p < 0.05$; **: $p < 0.01$.

Additionally, substrates may also play a vital role in the translocation and transformation of PPCPs by altering the rhizosphere solution mineralogy and electrochemistry (**Fig. 3**) and influencing the growth of plants (**Fig. 2**), which is supported by previous studies (Dhir, 2019; Miller et al., 2016). In vermiculite systems, PPCPs showed the highest propensity to accumulate in the tissues of non-inoculated plants, mainly because the increased biomass of *G. maxima* (**Fig. 5.2**) might promote the uptake and accumulation of PPCPs. To our knowledge, this study is the first one involving the uptake and translocation of these six PPCPs in the tissues of *G. maxima*. Nevertheless, relevant knowledge for the accumulation of those selected PPCPs in plant tissues is still lacking.

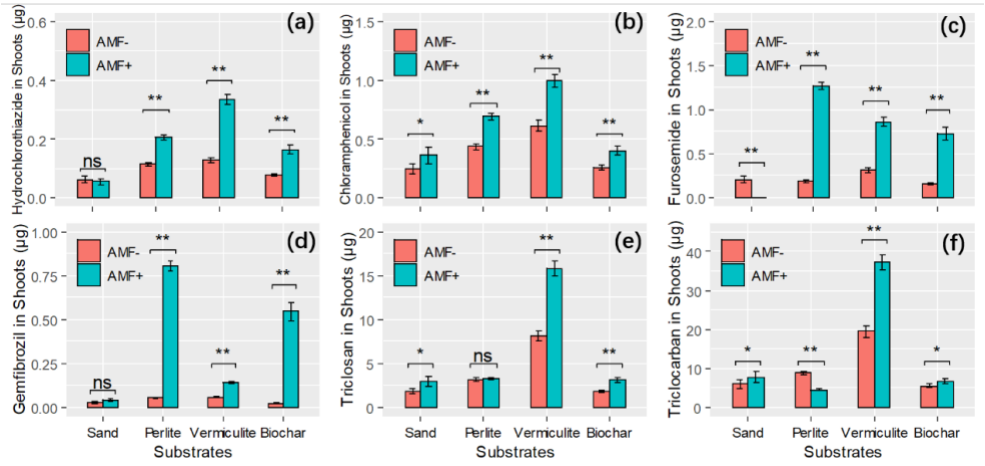


Fig. 5.5. The mass of PPCPs in plant shoots (µg). (a) hydrochlorothiazide; (b) chloramphenicol; (c) furosemide; (d) gemfibrozil; (e) triclosan; (f) triclocarban; The data are the means ± standard errors. ns: no differences; *: $p < 0.05$; **: $p < 0.01$.

Effect of AMF on the accumulation of PPCPs in plant tissues

In sand systems, the amount of hydrochlorothiazide, chloramphenicol, furosemide, gemfibrozil, triclosan, and triclocarban in the roots of inoculated plants were increased by 52.3%, 85.7%, 180.6%, 193.0%, 20.5%, and 11.2%, respectively than those in the non-inoculated controls. AMF significantly increased the uptake of PPCPs (except triclocarban) by plant roots ($p < 0.05$) (**Fig. 5.4**). In vermiculite systems, similarly, the amounts of chloramphenicol, furosemide, gemfibrozil, and triclosan in the roots of inoculated plants were significantly increased by 83.1%, 84.0%, 196.2%, and 66.7%, respectively, compared to the non-inoculated controls ($p < 0.001$) (**Fig. 5.4**). Noteworthy, the amounts of PPCPs in the shoots of inoculated plants in all substrate systems were higher than those in the non-inoculated controls, except furosemide in sand systems and triclocarban in perlite systems (**Fig. 5.5**). However, an opposite effect of AMF on the amount of PPCPs in plant roots was observed in perlite and biochar systems (**Fig. 5.4**). Compared to the non-inoculated controls, the amounts of chloramphenicol, triclosan, triclocarban in the roots of AMF inoculated *G. maxima* in perlite systems were significant ($p < 0.001$) decreased by 53.7%, 54.7%, and 49.4%, respectively. Similarly, in biochar systems, the amounts of hydrochlorothiazide,

chloramphenicol, furosemide, triclosan, and triclocarban in inoculated plants' roots were significantly reduced and lower than those of the non-inoculated controls ($p < 0.001$).

AMF could enhance PPCPs uptake by the roots of plants. Similar results also were observed in our previous study, revealing that the contents of ibuprofen and diclofenac in the roots of inoculated plants were 33.1% and 143.1% higher than that in the non-inoculated controls (Hu et al., 2021a). A possible explanation is that the extensive branched external mycelium developed by AMF symbiosis provides a mycorrhizal pathway to the host plants, thus improving the uptake and transport of PPCPs in plant tissues. By using a three-compartment system, Gao et al. (2010) demonstrated that the extraradical hyphae of AMF could extend into the PAHs-spiked soil to absorb and transfer PAHs (fluorene and phenanthrene) to the roots of plants (*Lolium multiflorum Lam.*) growing in uncontaminated soil. Our results showed that mycorrhizal inoculation had positive effects on the translocation of most PPCPs from roots to shoots and enhanced their accumulation in the shoots of *G. maxima* (**Fig. 5.4 and 5.5**). This could be attributed to the differences in plant species and compounds. It has been demonstrated that the accumulation and translocation of PPCPs in plant tissue vary among plant species and compounds (Dhir, 2019; Miller et al., 2016). Moreover, AMF showed positive effects on the accumulations of PPCPs in plant roots in sand and vermiculite systems. In contrast, negative effects of mycorrhizal inoculation on the accumulation of PPCPs in plant roots were observed in perlite and biochar systems (**Fig. 5.4**). A previous study indicated that zinc accumulation in inoculated plant tissues could be influenced by substrate texture, pH, and nutrients in the substrate (i.e., zinc and phosphorus deficiency) (Lehmann et al., 2014). Therefore, the effects of AMF on the accumulation in plant roots showed differences among the four substrate systems; it is mainly due to that the differences in substrate materials, plant growth (**Fig. 5.2**) and operating conditions (**Fig. 5.3**) could influence the translocation and transformation of PPCPs in inoculated plant tissues, thus resulting in positive/negative effects of AMF on the accumulation of PPCPs in plant roots. However, no study to date has examined the impact of substrates on the accumulation of PPCPs in AMF inoculated plant tissues. Knowledge about the mechanism of substrate influencing the accumulation of PPCPs in inoculated plant tissues is still unclear. Therefore, additional studies are necessary to reveal which role of substrates play in the effects of AMF on the uptake and transport of PPCPs in plants.

5.4.5 PPCPs removal in the aqueous phase

Effects of substrate on PPCPs removal

The concentration of each PPCP in the effluent of CWs was in the following order: sand > perlite > vermiculite > biochar (**SI, Fig. S5.5 and S5.6**). The best removal performance for PPCPs was found in biochar systems (more than 99.99%) (**Fig. 5.6**). On the contrary, sand systems showed the lowest removal capacity of PPCPs, with removal efficiencies of 57.7% for hydrochlorothiazide, 83.7% for chloramphenicol, 68.6% for furosemide, 64.1% for gemfibrozil, 99.8% for triclosan, and 99.5% for triclocarban, respectively. Perlite and vermiculite systems showed more effective removal efficiencies on PPCPs than sand systems. The removal efficiencies of hydrochlorothiazide, chloramphenicol, furosemide, gemfibrozil, triclosan, and triclocarban were increased by 18.2-22.5%, 10.9-11.9%, 21.8-22.7%, 20.8-23.4%, 0.40-0.41%, and 0.20-0.21%, respectively (**Fig. 5.6**). Two-way ANOVA analysis indicated that substrate significantly affected the removal of all PPCPs in CWs ($p < 0.01$) (**SI, Table S5.4**).

The removal efficiency of PPCPs in adsorptive substrate systems was significantly higher than that in sand systems. A possible explanation could be that these three substrates have a higher adsorption capacity of PPCPs than sand (Deng et al., 2021; Dordio and Carvalho, 2013; Dordio et al., 2009), which could contribute to absorb more PPCPs from the aqueous phase and retain inside the CWs. Meanwhile, the porous structures and large surface area of adsorptive substrates can provide more microbial attachment interfaces (**Fig. 5.1**), which could benefit the growth and development of microbes and the adsorption of PPCPs (Deng et al., 2021), thus enhancing the biodegradation of PPCPs. These results are in accordance with findings reported by Wirasnita et al. (2018), who investigated the effects of activated carbon on the removal of organic contaminants (bisphenol A, bisphenol F, bisphenol S and 4-tert-butylphenol) in CWs and demonstrated that bacterial populations in activated carbon system were one-two orders higher than those in the normal system, thus resulting in a significantly higher removal performance of the selected organic contaminants in activated carbon system. Another reason for the higher removal efficiency of PPCPs in perlite, vermiculite and biochar systems could be that the application of adsorptive substrates improved the operation conditions of CWs (**Fig. 5.3**), especially the dissolved oxygen

(**Fig. 5.3a**). The increased oxygen could favor the aerobic biodegradation process, which might also be responsible for improving the biodegradation of PPCPs in CWs (Ilyas and Van Hullebusch, 2020). Similarly, Huang and Gu (Huang and Gu, 2019) also found that biochar media could promote root aerenchyma tissues and macrophyte porosity in CWs to allow an increase in root oxygen loss, thus enhancing aerobic microbial metabolisms, such as nitrification, methane oxidation and organic degradation. In addition, the effects of substrate on plant growth may also influence PPCPs removal in CWs (Deng et al., 2021). As shown in **Fig. 5.2**, the improved plant growth was found in perlite, vermiculite and biochar systems, which could be beneficial for PPCPs removal through plant uptake, oxygen release, organic rhizodeposition produces (e.g., exudates, mucigels, and dead cell materials) and the provision of more sites for microorganisms (Deng et al., 2021; Dordio and Carvalho, 2013). Overall, applying absorptive substrates (such as perlite, vermiculite, and biochar) could enhance PPCPs removal in CWs due to their adsorption capacity and the positive effects on plant growth and microbes.

Effects of AMF on PPCPs removal

AMF significantly affected the removal of chloramphenicol, gemfibrozil, triclosan, and triclocarban in sand systems ($p < 0.01$) (**Fig. 5.6**). However, insignificant effects of AMF on the removal of PPCPs can be found in other systems. Especially in vermiculite systems, the removal of furosemide under AMF+ treatments was even lower (2.7%) than that under AMF- treatments.

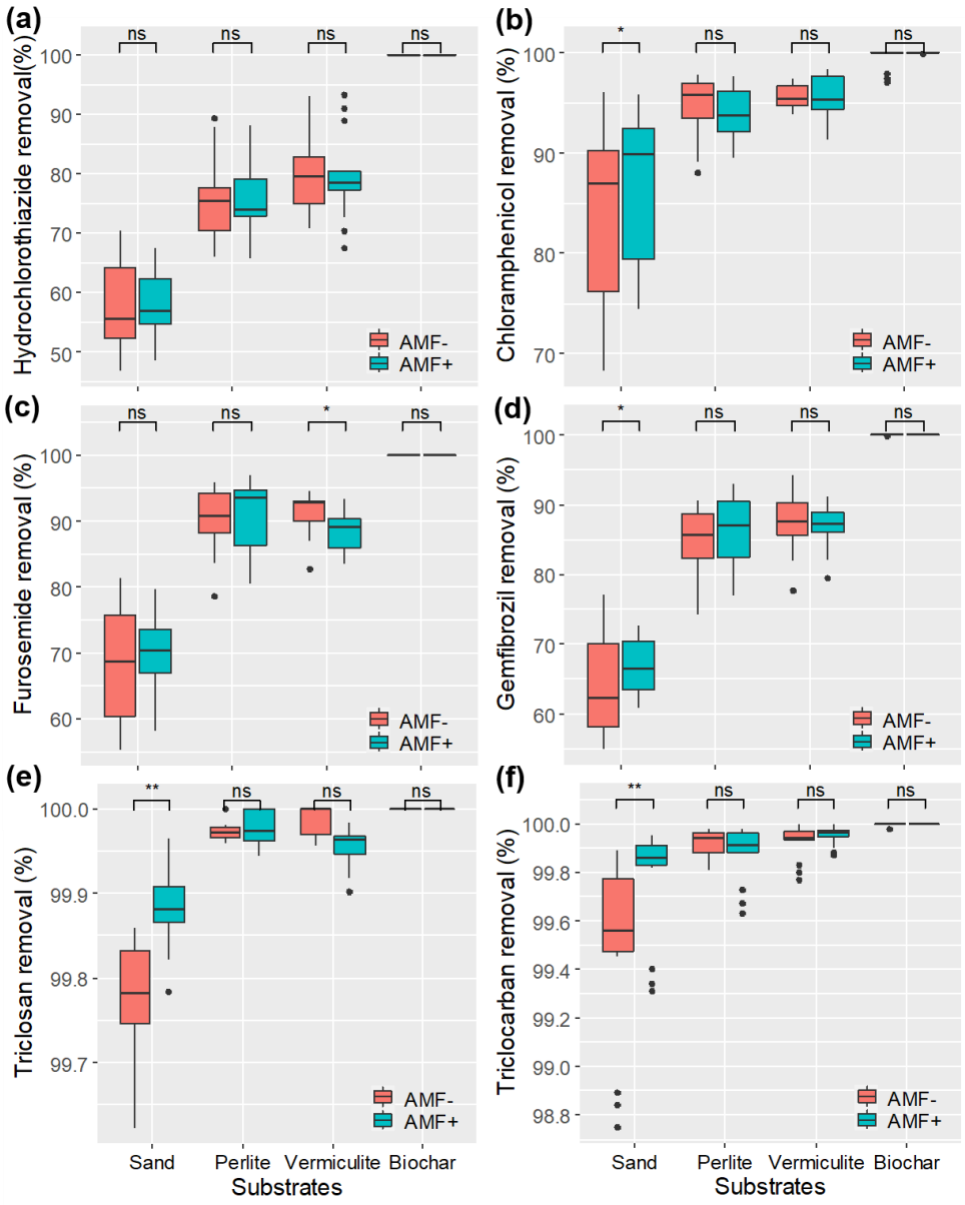


Fig. 5.6. Mass removal efficiency of PPCPs in different substrate CW systems with AMF- and AMF+ treatments. (a) Hydrochlorothiazide, (b) Chloramphenicol, (c) Furosemide, (d) Gemfibrozil, (e) Triclosan and (f) Triclocarban. The data are the means ± standard errors. ns: no differences; *: $p < 0.05$; **: $p < 0.01$.

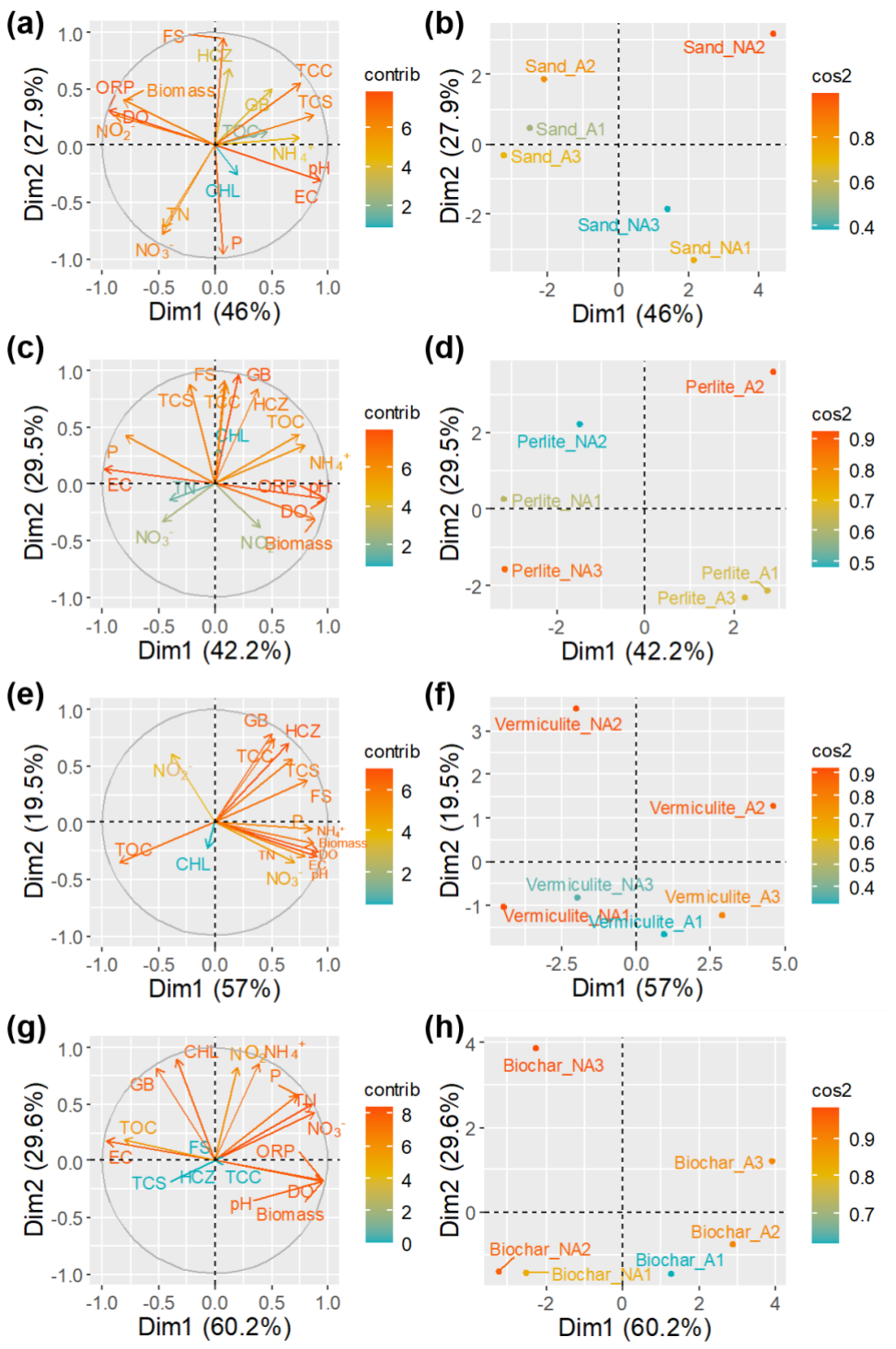

(a)
Dim2 (27.9%)
Dim1 (46%)
FS
HCZ
TCC
ORP
Biomass
GB
TCS
DO
NO2
TOC
NH4+
pH
CHL
EC
TN
NO3-
P
contrib
(b)
Sand_NA2
Sand_A2
Sand_A1
Sand_A3
Sand_NA3
Sand_NA1
cos2
(c)
Dim2 (29.5%)
Dim1 (42.2%)
FS
GB
TCS
TCC
HCZ
TOC
CHL
P
NH4+
EC
TN
ORP
pH
DO
NO3-
NO2
Biomass
contrib
(d)
Perlite_A2
Perlite_NA2
Perlite_NA1
Perlite_NA3
Perlite_A1
Perlite_A3
cos2
(e)
Dim2 (19.5%)
Dim1 (57%)
GB
HCZ
NO2
TCC
TCS
FS
P
NH4+
TOC
Biomass
DO
CHL
TN
EC
NO3-
pH
contrib
(f)
Vermiculite_NA2
Vermiculite_A2
Vermiculite_NA3
Vermiculite_A3
Vermiculite_NA1
Vermiculite_A1
cos2
(g)
Dim2 (29.6%)
Dim1 (60.2%)
CHL
NO2
NH4+
GB
P
TN
TOC
NO3-
FS
ORP
EC
TCS
HCZ
TCC
DO
pH
Biomass
contrib
(h)
Biochar_NA3
Biochar_A3
Biochar_A2
Biochar_NA2
Biochar_A1
Biochar_NA1
cos2

Fig. 5.7. Principal component analysis (PCA) of biomass, operation conditions, conventional pollutants and selected PPCPs in effluents of CW systems, and the scores of each treatment on Dim 1 and Dim2. (a) and (b): sand systems; (c) and (d): perlite systems; (e) and (f): vermiculite systems; (g) and (h): biochar systems. CHL, HCZ, FS, GB, TCS, and TCC represent the six selected PPCPs, including chloramphenicol, hydrochlorothiazide, furosemide, gemfibrozil, triclosan, and triclocarban, respectively. TOC, TN and P represent total organic carbon and total nitrogen and phosphate, respectively. A1, 2 and 3 represent the triplicates of AMF+ treatments; NA1, 2 and 3 represent the triplicates of AMF- (Non-AMF) treatments, respectively.

AMF enhanced the removal efficiency of PPCPs from the aqueous phase of sand systems. A possible explanation is that AMF promoted the accumulation of PPCPs in plant tissues (**Fig. 5.4 and 5.5**) and the rhizosphere soil (**Table 5.1**). Similar results were also reported in our previous study, indicating that the positive effects of AMF on pharmaceuticals removal could attribute to the increase in their accumulation in plant roots and rhizosphere soil (Hu et al., 2021a). Moreover, the positive effects of AMF on plant growth (**Fig. 5.2**) and the oxygen conditions (**Fig. 5.3a**) might also contribute to the development of aerobic microorganisms, and thus inducing various biological processes to enhance the elimination of pollutants in CWs, including PPCPs (Dordio and Carvalho, 2013; Keerthanan et al., 2021). This was supported by the results of principal component analysis (PCA), indicating that the operating parameters among the four substrate systems, including plant growth, operating conditions, and the concentrations of conventional pollutants and PPCPs in the effluents, showed significant differences between AMF- and AMF+ treatments (**Fig. 5.7**). However, in our study, there were insignificant differences in PPCPs removal between AMF- and AMF+ treatments in adsorptive substrate systems. A possible reason is that the application rate of the adsorptive substrate (100%) may potentially inhibited the performance of AMF on PPCPs removal. Conversa et al. (2015) found that high application rate of biochar (70%) could have negatively affected plant growth through osmotic stress and/or the inhibition of mycorrhizal activity. They suggested that the low application rate of biochar (< 30%) was more favorable to plant growth and AMF development. Meanwhile, another possible explanation for the insignificant effects of AMF on PPCPs removal in the adsorptive substrate systems is that the residence time (only two hours per cycle) may be too short for PPCPs to quickly eliminate from the aqueous phase of CWs through biodegradation or plant uptake (Dordio and Carvalho, 2013; Wu et al., 2015a). As shown in **SI, Table S5.1**, the biological half-life of the selected PPCPs indicated their environmental persistence, ranging from 0.97 d to more than 100 d. microbial degradation of PPCPs. Consequently, the adsorption of substrates and the attached microbes could play dominant roles in PPCPs removal from the

aqueous phase in perlite, vermiculite, and biochar systems. This result ties well with a previous study reported by Wirasnita et al. (2018), who points out that the removal of organic contaminants in adsorptive substrate systems is mainly through two steps, initially adsorbed by substrate then degraded by attached-microorganisms. Therefore, the type of substrate might be the decisive factor affecting PPCPs removal in tidal flow CWs, especially the short residence time of wastewater in the present study. By contrast, the role of AMF on PPCPs in perlite, vermiculite and biochar systems could be considered relatively unimportant. This mainly because the effects of adsorptive substrates (perlite, vermiculite and biochar) on plant growth (**Fig. 5.2**) and operating conditions (**Fig. 5.3**) were more significant than that of AMF in this study. These were confirmed by the results of cluster analysis, indicating that the differences of operating parameters among the four substrate systems, including biomass, operation conditions (pH, ORP, DO, and EC), conventional organic pollutants (TOC, P, TN, NH_4^+-N, NO_3^--N, and NO_2^--N) and the concentrations of the selected PPCPs in the effluent, were significantly higher than those between AMF+ and AMF- treatments (**SI, Fig. S5.7**).

It is well known that PPCPs removal in CWs occurs through a series of complex physical, chemical, and microbial interactions (Vymazal et al., 2017), and involves a variety of processes, including plant uptake, microbial degradation, adsorption/desorption, photolysis, hydrolysis and volatilization. Our study suggested that AMF might play an important role in affecting the removal pathways of PPCPs in CWs, and this ability could be affected by the type of substrates. Meanwhile, due to the short residence time of wastewater in CWs, the predominant removal process of PPCPs in the present study could be attributed to the adsorption of substrates and the attached microbes. Consequently, AMF had positive effects on removing PPCPs in sand systems but insignificant effects in perlite, vermiculite, and biochar systems. However, the mechanisms by which AMF affects PPCPs removal in CWs are not fully understood. Future research should consider the potential effects of AMF on PPCPs metabolism in CW systems more carefully, for example, the role of AMF in the metabolic pathway of PPCPs, including plant uptake, microbial degradation, and absorption by substrate and attached microbes. Besides, more suitable hydraulic conditions (e.g., a long-term flooding condition or a more frequent water change cycle of tidal flow) should be considered in the following experiment to approach the need of actual engineering applications.

5.5 Conclusion

AMF symbiosis had a better performance in perlite, vermiculite, and biochar systems than sand systems. The presence of AMF in CWs enhanced the growth of G. *maxima*. Substrates and AMF affected the accumulation of PPCPs in the rhizosphere soil of CWs. Hydrochlorothiazide, chloramphenicol, furosemide, and gemfibrozil were mainly accumulated in the roots, but the amounts of triclosan and triclocarban in plant shoots were 1.4-2.5 and 5-7.9 times higher than that in roots, respectively. Moreover, the accumulations of PPCPs in the roots of inoculated plants were increased by 21-193% and 67-196% in sand and vermiculite systems but decreased 13-55% and 51-100% in perlite and biochar systems, respectively, compared to the non-inoculated controls. AMF enhanced the translocation of PPCPs to plant shoots in CWs, resulting in a higher amount of PPCPs in the shoots of inoculated plants than that of non-inoculated plants. Additionally, AMF positively affects PPCPs removal in sand systems, while insignificant differences of PPCPs removal between AMF- and AMF+ treatments were observed in perlite, vermiculite, and biochar systems. These results suggested that AMF might have the potential to enhance PPCPs removal in CW systems. Overall, our results provide a good starting point for applying AMF in the phytoremediation of PPCPs in real-scale wetland systems. However, additional studies are necessary to investigate the effects of AMF on the composition of microbial community and the biodegradation of PPCPs in CWs to get a better insight into the functional roles of AMF for PPCPs removal.

5.6 Supplementary Materials

Methods and materials

- The introduction of the selected substrates

Tables:

- Table S5.1. Biological half-life of the selected PPCPs in the environment
- Table S5.2. The actual concentration of PPCPs in the environment
- Table S5.3. Physiochemical capacities of PPCPs
- Table S5.4. Two-way ANOVA analysis of PPCPs accumulation in plant tissues

Figures:

- Fig. S5.1. The composition of AMF- and AMF+ treatment systems
- Fig. S5.2. AMF colonization in the roots of *G. maxima* from different substrate systems
- Fig. S5.3. Mycorrhizal status in the roots of *G. maxima* in different CW systems
- Fig. S5.4. The temperature changes during the experiment.
- Fig. S5.5. The concentrations of PPCPs (hydrochlorothiazide, chloramphenicol, furosemide, and gemfibrozil) in the effluent of different substrate system
- Fig. S5.6. The concentrations of PPCPs (triclocarban and triclosan) in the effluent of different substrate systems
- Fig. S5.7. The cluster analysis of the operation parameters in different substrate CWs
- Fig. S5.8 The temporal changes of operating conditions in different systems with AMF- and AMF+ treatments.

Methods and materials:

The introduction of the selected substrates:

The characteristics of substrates were measured by Scanning Electron Microscopy with Energy Dispersive X-Ray Analysis (SEM-EDX, Tescan VEGA3 XMU), as shown in **Fig. 5.1**. Sand is made up of two main elements: Si and O, which is a chemically inert and relatively hard mineral. It was obtained from Sklopísek Střeleč, s.r.o., Czech Republic (https://en.glassand.eu). Perlite is a glassy volcanic rock, which is essentially a metastable amorphous aluminum. Expanded perlite likes little balls with a soft honeycomb texture inside. As perlite contains a high silica content, it is chemically inert in many environments and hence is also an excellent filter aid and filler in various industrial processes (Dordio et al., 2017). The basic elements in perlite include O, Na, Mg, Al, Si, K, Ti and K. It was obtained from AAA Komíny s.r.o., Czech Republic (https://www.aaakominy.cz). Vermiculite is a hydrated magnesium iron aluminum silicate mineral in the form of shiny flakes, which are usually golden brown to blackish in color. The basic elements of vermiculite include O, Na, Mg, Al, Si, K, Ca, Ti, Cr and Fe. Vermiculite is expanded by heating the crude flaky mineral. The expanded granules are many times greater in volume than before heating and are more concertina shaped, more golden to light, greyish brown sometimes silvery gold in color and are much less dense. Expanded vermiculite has unique and versatile characteristics; it is lightweight, provides energy savings when used as insulation, it is non-combustible, highly absorbent, pH neutral, inert, non-reactive to all but very strong acids and compressible (Wen et al., 2016). Vermiculite was obtained from Robimaus s.r.o., Czech Republic (https://www.robimaus.cz). Biochar, a porous carbon-rich material produced from biomass under oxygen-free or oxygen-limited conditions, is recently emerging as an innovative and promising additive in CWs that exhibits high potential in enhancing treatment performance of PPCPs (Deng et al., 2021; Huang and Gu, 2019). Carbon is the main element in biochar, O, Mg, Si, S, Cl, K, Ca and Mn are the trace elements in biochar. It was obtained from Institute of Experimental Botany of the Czech Academy of Sciences, Czech Academy of Sciences, Czech Republic. Biochar was produced by spruce wood in a muffle furnace. Wood was quickly heated at 600 ^{o}C (fast pyrolysis), then slowly cool down under 16.7 mL/min nitrogen flow rate at atmospheric pressure with the retention time of 30 min (slow pyrolysis).

Tables:

Table S5.1. Biological half-life of the selected PPCPs in the environment.

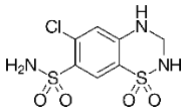
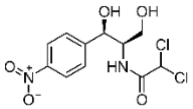
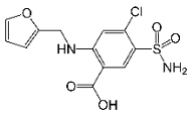
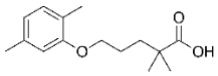
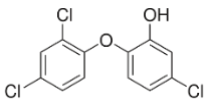
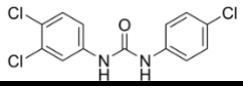
PPCPs	Biological half-life in the environment (d)	
	Aquatic environments	Soil environments
Hydrochlorothiazide	NO data	11-18 aerobic conditions (Biel-Maeso et al., 2019) 28.8-84.5 (fitted by 3 models) (Radke and Maier, 2014)
Chloramphenicol	NO data	Aerobic: 43.3 (sterilized) 6.70 (non-sterilized); anaerobic: 53.3 sterilized 8.60 non-sterilized (Pan and Chu, 2016); 0.97-2.05 in three soils, 0.97-14.52 in soil with different treatments and different concentrations (Zhang et al., 2013)
Furosemide	NO data	NO data
Gemfibrozil	10-19 estuarine waters (Aminot et al., 2018)	3-4 aerobic conditions (Biel-Maeso et al., 2019)
Triclosan	< 10 (two special river) 90 (in winter) (Tixier et al., 2002)	187±6 (experimental) and 120 (predicted) (Walters et al., 2010); 20-58 (Wu et al., 2009a); 18 (Ying et al., 2007); 18

PPCPs	Biological half-life in the environment (d)	
	Aquatic environments	Soil environments
	60 (estimated using QSAR analysis) (Halden and Paull, 2005)	(aerobic soil) (Chenxi et al., 2008); 120 (estimated using QSAR analysis) (Halden and Paull, 2005)
Triclocarban	60 (estimated using QSAR analysis) (Halden and Paull, 2005)	13.59 (100 ug/kg) 19.97 (1000 ug/kg) (Xu et al., 2009) >1000 (experimental) and 120 (predicted) (Walters et al., 2010) 87-231 (Wu et al., 2009a) 108 (Ying et al., 2007) 120 (estimated using QSAR analysis) (Halden and Paull, 2005)

Table S5.2. The actual concentration of PPCPs in the environment.

PPCPs	Detected concentration in the environment (ng/L)
Hydrochlorothiazide	**2800** (effluent, WWTP, US) (Kostich et al., 2014); **1261-17589** (rivers in Spain) (Valcárcel et al., 2011)
Chloramphenicol	**4.18-28.36** (Jiang et al., 2011)
Furosemide	**388-3228** (Valcárcel et al., 2011)
Gemfibrozil	**1113-5192** (Valcárcel et al., 2011)
Triclosan	< **5-195** (rivers in UK) (Kasprzyk-Hordern et al., 2008); **42-213** (wastewater effluents) and **11−98** (in the receiving rivers). (Singer et al., 2002); **586** in raw wastewater. (Ying and Kookana, 2007); **0-478** (water bodies, rivers in China) and **0-1329** (sediments, rivers in China) (Zhao et al., 2010)
Triclocarban	**0-338** (water bodies, rivers in China) and **0-2633** (sediments, rivers in China) (Zhao et al., 2010); **2-250** (Surface water downstream of WWTPs, US) and **750-25900** (Primary sludges obtained from select US wastewater treatment plants) (Gautam et al., 2014)

Table S5.3. Physiochemical capacities of PPCPs.

PPCPs	Structure	Molecular Weight	Solubility	$LogK_{ow}$	pK_a
Hydrochlorothiazide		297.7	722	-0.07	7.9
Chloramphenicol		323.13	2500	1.14	9.61
Furosemide		330.74	73.1	2.03	3.9
Gemfibrozil		250.33	11	4.77	4.5
Triclosan		289.5	10	4.76	7.9
Triclocarban		315.6	0.11	4.90	12.7

Date sources were collected from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Unit: Molecular Weight: g/mol; Solubility: mg/L in water.

Table S5.4. Two-way ANOVA analysis of plant growth, PPCPs removal and PPCPs accumulation in plant tissues. Substrates and AMF treatments as main factors and substrates * AMF treatments as interaction effect. *: $p < 0.05$; **: $p < 0.01$.

Parameters	Substrates	AMF	Substrates*AMF
Plant growth			
Shoot height	**	**	**
Root length	**	**	**
PPCPs removal in the aqueous phase			
Hydrochlorothiazide	**	0.84	0.99
Chloramphenicol	**	0.26	0.40
Furosemide	**	0.86	0.31
Gemfibrozil	**	0.26	0.39
Triclosan	**	**	**
Triclocarban	**	*	**
PPCPs in plant's roots			
Hydrochlorothiazide	**	**	**
Chloramphenicol	**	**	**
Furosemide	**	**	**
Gemfibrozil	**	**	**
Triclosan	**	**	**
Triclocarban	**	**	**
PPCPs in plant's shoots			
Hydrochlorothiazide	**	**	**
Chloramphenicol	**	**	**
Furosemide	**	**	**
Gemfibrozil	**	**	**
Triclosan	**	**	**
Triclocarban	**	**	**

Figures:

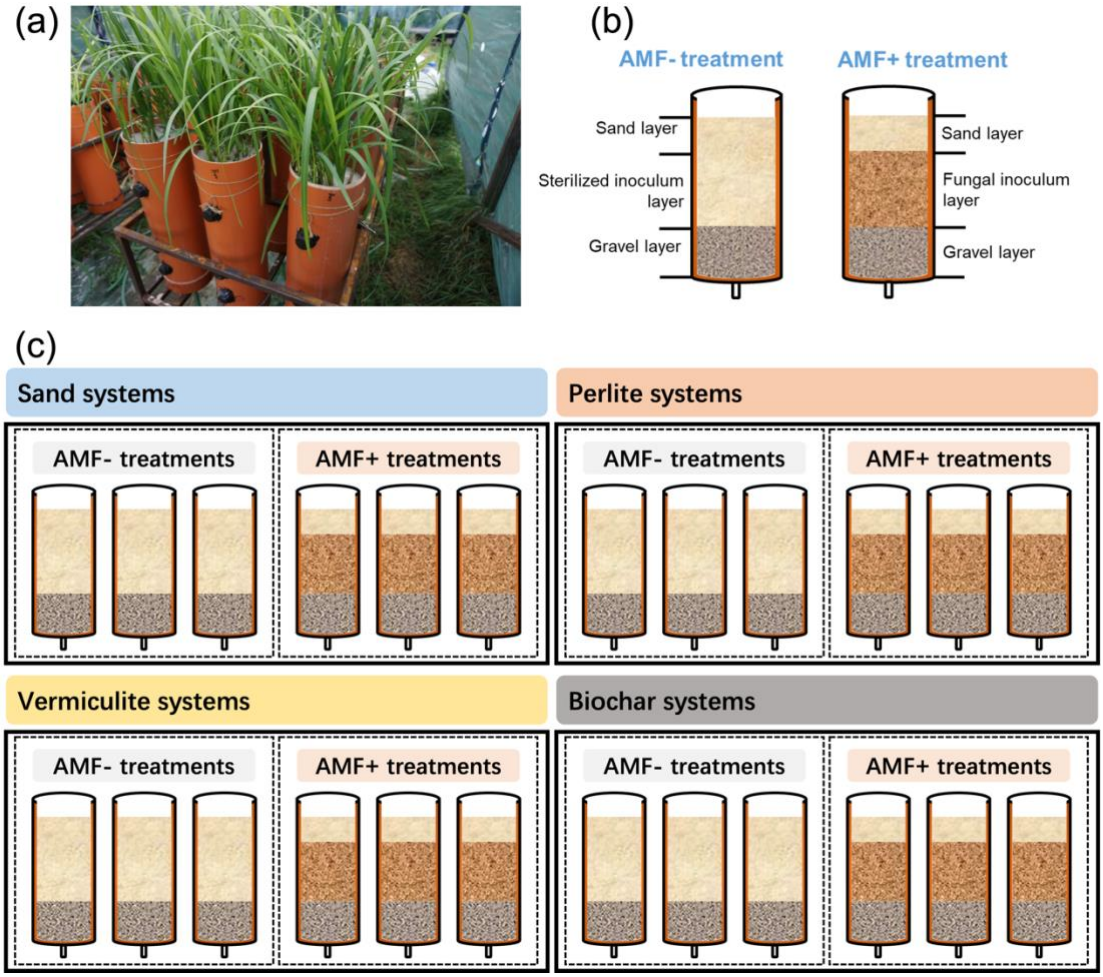


Fig. S5.1. (a). The real CW systems in this study. (b). The composition of AMF- and AMF+ treatment systems. (c). Sketch of four CWs filled with different substrate. Each column was divided into parts: (1) 150 mm bottom layer filled with gravel; (2) 200 mm middle layer filled with different substrates (sand, perlite, vermiculite, and biochar) in different substrate CW systems; (3) 150 mm top layer covered with sand. In AMF+ treatments, 350g fungal inoculum was added and mixed with the middle layer substrates. In AMF- treatments, 350 g sterilized fungal inoculum together with 100 mL inoculum filtrate (Wu et al., 2018) were added into the middle layer substrates to provide a similar microflora except for the absence of AMF.

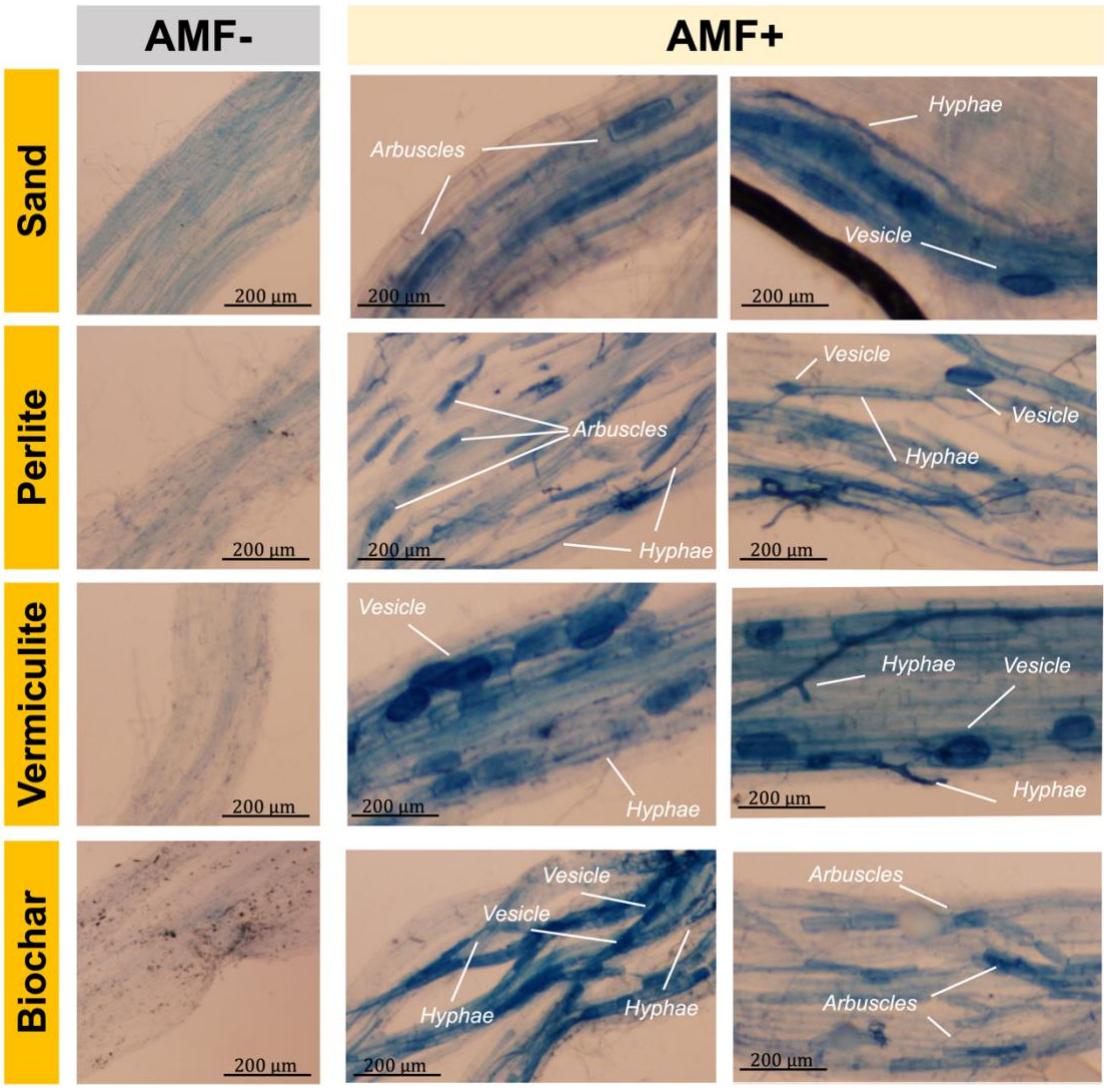


Fig. S5.2. AMF colonization in the roots of *G. maxima* from different substrate systems. These images were obtained from a microscopy (magnification: 40 x, Olympus BX41 Phase Contrast & Darkfield Microscope).

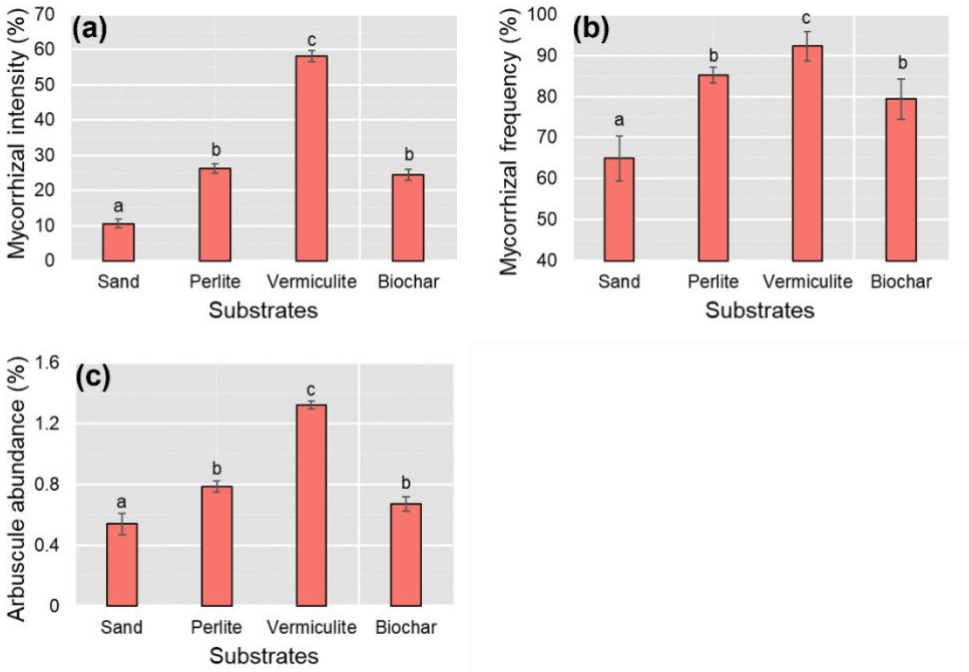


Fig. S3. Mycorrhizal status in the roots of *G. maxima* in different CW systems. (a). The intensity of mycorrhizal inoculation; (b). The frequency of mycorrhiza; (c). The arbuscule abundance.

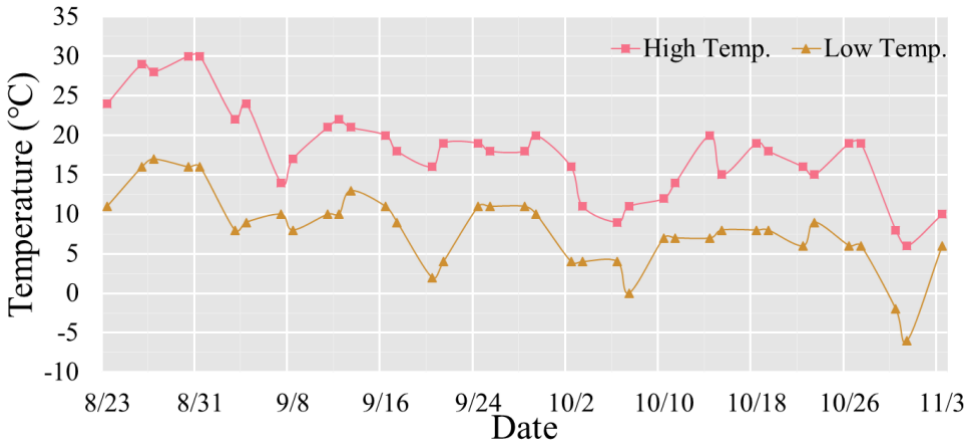


Fig. S5.4. The temperature (Temp.) changes during the experiment.

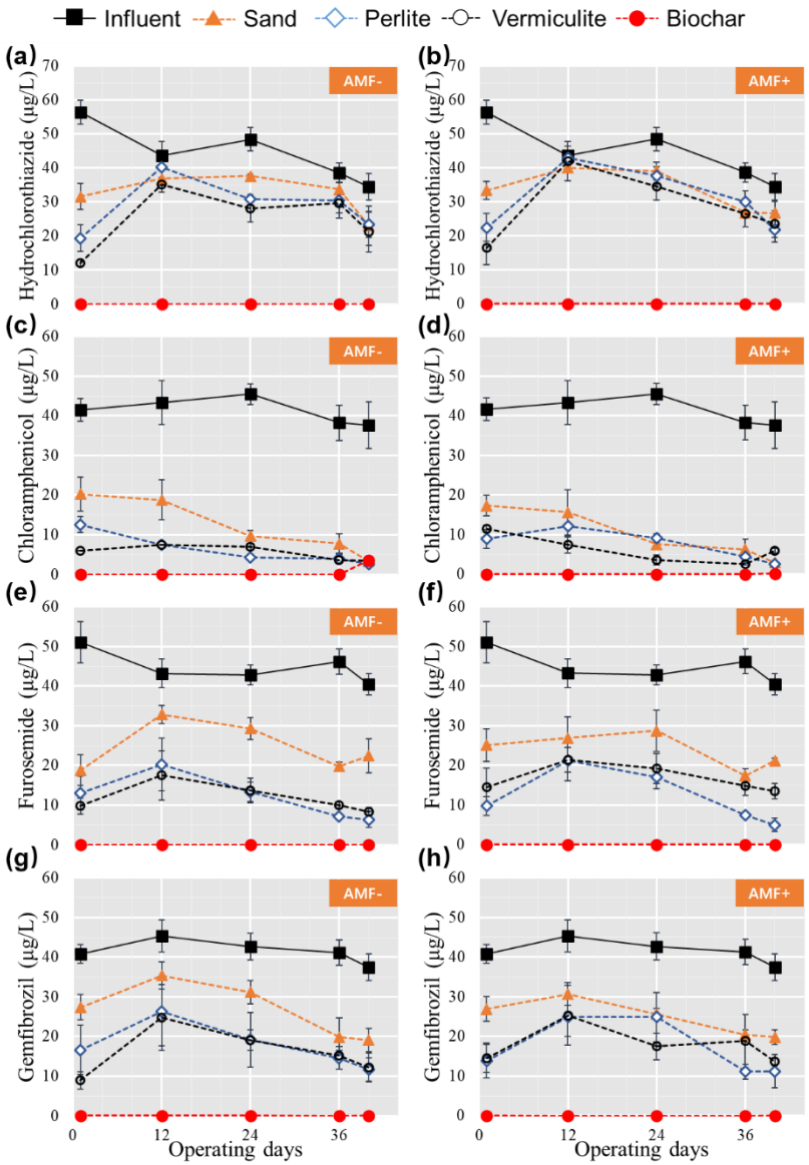


Fig. S5.5. The concentrations of PPCPs in the effluent of different substrate systems. Hydrochlorothiazide in AMF- (a) and AMF+ (b) treatments; Chloramphenicol in AMF- (c) and AMF+ (d) treatments; Furosemide in AMF- (e) and AMF+ (f) treatments; Gemfibrozil in AMF- (g) and AMF+ (h) treatments. The data are the means ± standard error.

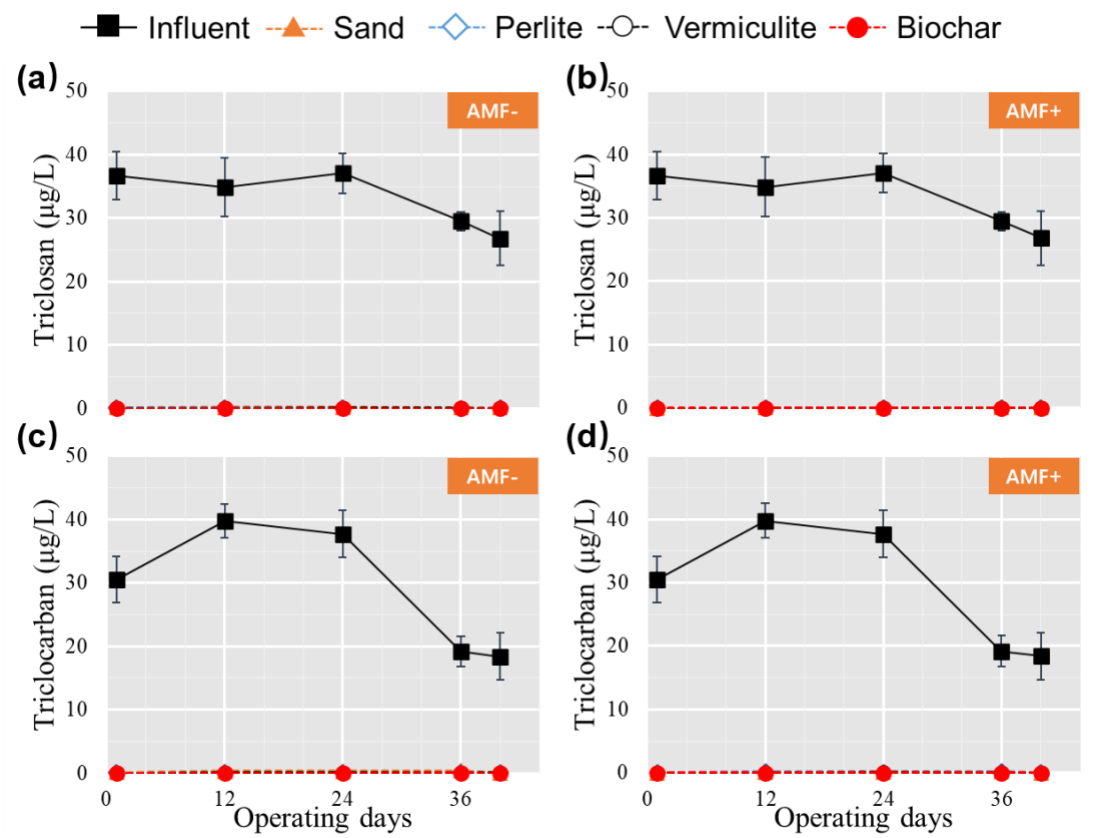


Fig. S5.6. The concentrations of PPCPs in the effluent of different substrate systems after the addition of PPCPs. Triclosan in AMF- (a) and AMF+ (b) treatments; Triclocarban in AMF- (c) and AMF+ (d) treatments. The data are the means ± standard errors.

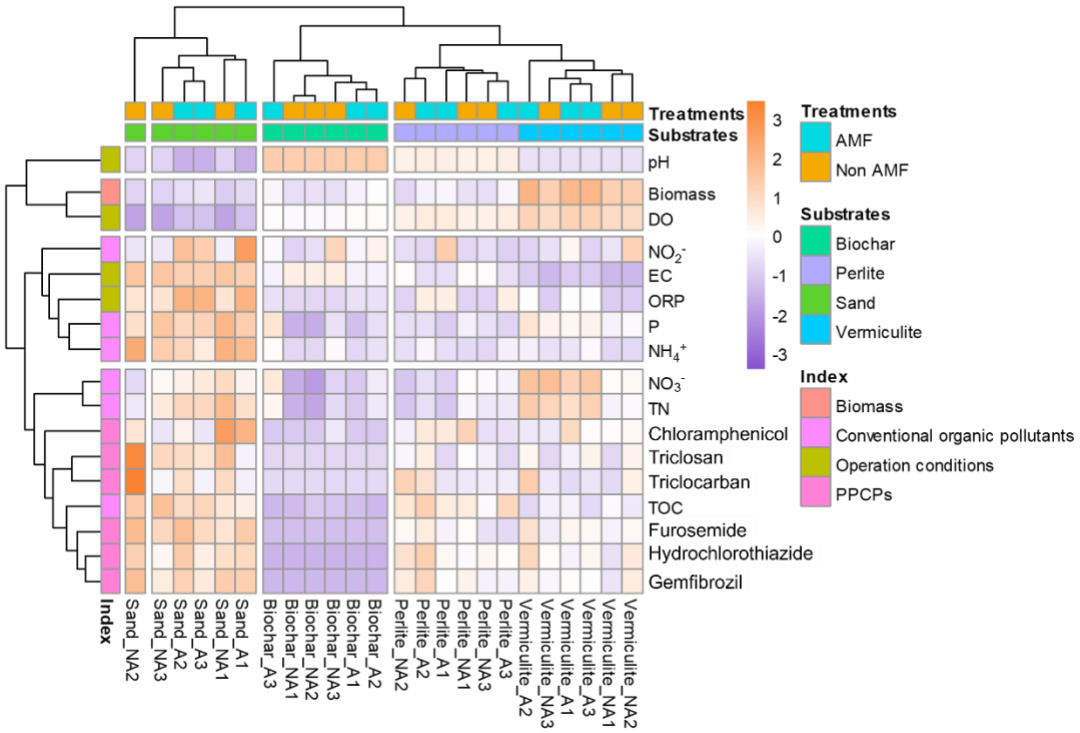


Fig. S5.7. The cluster analysis of biomass, operation conditions (pH, ORP, DO, and EC), conventional organic pollutants (TOC, P, TN, NH_4^+-N, NO_3^--N, and NO_2^--N) and selected PPCPs in the effluent of the four substrate systems with AMF- and AMF+ treatments. Colors in the heatmap indicate the correlation between the different data sets. TOC, TN and P represent total organic carbon and total nitrogen and phosphate, respectively. A1, 2 and 3 represents the triplicates of AMF+ treatments；NA1, 2 and 3 represents the triplicates of AMF- (Non-AMF) treatments, respectively.

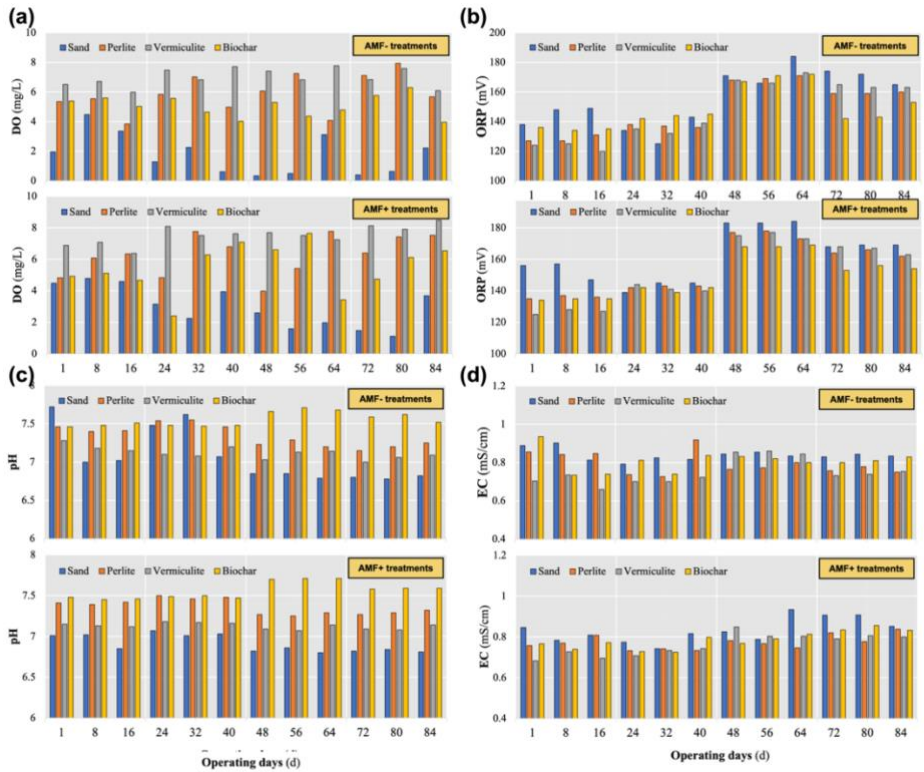


Fig. S5.8. The temporal changes of operating conditions in different systems with AMF- and AMF+ treatments. (a). DO; (b). ORP; (c). pH; (d). EC. The data is the mean value of three replicates.

Chapter VI

Performance of constructed wetlands for the purification of wastewater containing PPCPs: Effects of substrate, plant, and arbuscular mycorrhizal fungi

Bo Hu, Shanshan Hu, Jan Vymazal, Zhongbing Chen

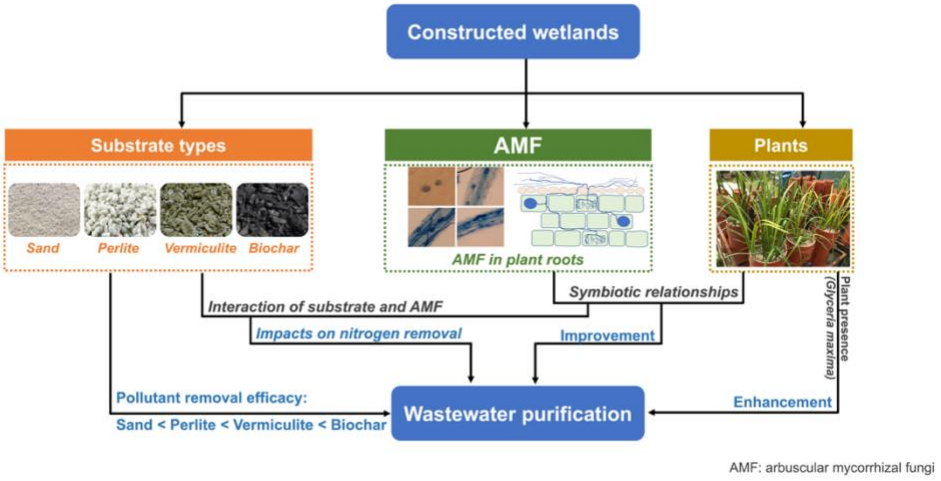
Submitted to Process Safety and Environmental Protection

Contents

6.1 Abstract

This study focused on the pharmaceuticals and personal care products (PPCPs) containing wastewater purification in arbuscular mycorrhizal fungi (AMF) assistant constructed wetlands (CWs) with adding different substrates. Results showed that the removal efficiencies of total organic carbon (TOC), phosphate (PO_4^{3-}-P), ammonium (NH_4^+-N), and total nitrogen (TN) in adsorptive substrate (perlite, vermiculite, or biochar) systems were 3.8-11.4%, 11.6-30.6%, 16.5-31.2%, and 6.2-34.6% higher than those in the sand systems, respectively. Meanwhile, the removal efficiencies of TOC, PO_4^{3-}-P, and TN in CWs with different substrates were in the following order: sand < perlite < vermiculite < biochar. AMF increased TOC, TN, and PO_4^{3-}-P removal in CWs, but the adsorptive substrates showed more significant influences on wastewater purification than AMF. Besides, wastewater purification was also improved in planted (*Glyceria maxima*) CWs. Therefore, these results indicated that the interaction effects of adsorptive substrates and AMF could enhance the removal performance of pollutants from wastewater in planted CWs.

Graphical abstract:



6.2 Introduction

Constructed wetlands (CWs) have been wildly adapted for pharmaceuticals and personal care products (PPCPs) containing wastewater removal because of the advantages in low energy requirements and easy operation and maintenance (Hu et al., 2021c). However, wetland plants and microbes are frequently exposed to the stress of PPCPs, which may also affect plant growth and microbial activities as well as their subsequent performance to remove pollutants (Hu et al., 2021c; Ji et al., 2022). For example, PPCPs might reduce photosynthetic pigments in plants, inhibit root development, and reduce mature leaves' number and size (Bartrons and Peñuelas, 2017). Díaz-Cubilla et al. (2022) revealed that PPCPs could produce microbial stress and irreversible cell damage of microbes, influencing the anaerobic digestion process of anaerobic bioreactor and decreasing the removal efficiency of organic matters. Therefore, there is an urgent need to enhance plant tolerance to PPCPs stress, thus improving the performance and sustainability of CWs.

Arbuscular mycorrhizal fungi (AMF) are essential components of soil microorganisms, which can establish a mutualistic symbiosis with the roots of most terrestrial plant species (nearly 85%) in the terrestrial ecosystems (Smith and Read, 2008). The plant-mycorrhizal association can develop the extraradical mycelium beyond the root-hair zone and establish tree-shaped subcellular structures within root cells, thus, improving nutrient acquisition and plant tolerance to environmental stresses, including drought, cold, salinity, and organic contaminants (Ajit Varma, 2017; Hu et al., 2020a). In addition to providing these benefits for the host plants, AMF also plays a significant role in boosting the development of rhizosphere microorganisms by stimulating the production of root exudates, phytoalexins, and phenolic compounds (Latef et al., 2016; Toljander et al., 2007). For these reasons, AMF has been regarded as a critical phytoremediation approach to re-establish the degraded ecosystems, such as contaminated soils, abandoned agricultural fields, and grassland (Ajit Varma, 2017).

Nowadays, numerous pieces of evidence have demonstrated that the symbiotic relationships between AMF and plant roots can be found from various wetland habitats, including fens, swamps, marshes, shorelines, bays, floating wetland mats, and natural wetlands (Fusconi and Mucciarelli, 2018; Huang et al., 2021; Ramírez-Viga et al., 2020; Wang et al., 2018a). Commonly, AMF colonization in the roots of wetland plants

is invariably lower than that of terrestrial plants. However, recent evidence has proven that AMF can also provide significant advantages for plants in wetland systems (Xu et al., 2016). Palacios et al. (2021) investigated AMF inoculation in two plant species (*Ficinia nodosa* and *Carex appressa*) grown in stormwater biofilters, suggesting the positive effects of AMF on plant growth could directly improve nitrogen, phosphorus and Cr removal from stormwater, leading to a better performance of biofilters. Recently, our study evaluated the effects of AMF on PPCPs removal in CWs with different substrates (Hu et al., 2022). The results showed that AMF had positive impacts on PPCPs removal in sand systems but insignificant effects in perlite, vermiculite, or biochar systems, suggesting that the type of substrate may influence the role of AMF in PPCPs removal. However, few studies have focused on the combined impacts of substrate and AMF on the performance of CWs for treating PPCPs contaminated wastewater.

Therefore, this study aimed to investigate: (1) the effects of vegetation and substrates on pollutant removal in CWs; (2) the impacts of AMF on purification of pollutants in CWs; (3) the influence of interaction between substrates and AMF on PPCPs containing wastewater purification in CWs.

6.3 Materials and methods

6.3.1 Chemicals and materials

Glyceria maxima were selected as the wetland plant in this study because it is a common wetland species in Europe. The seedlings of *G. maxima* were collected from a natural pond on the campus of the Czech University of Life Sciences Prague. Plant roots were surface-sterilized according to the protocol described in our previous study (Hu et al., 2022). *Rhizophagus irregularis* (BEG140) was selected as the AMF inoculum, obtained from the Institute of Botany, Czech Academy of Science (Prague, Czech Republic). Sand (0.1-0.6 mm), expanded perlite (0.1-2.0), expanded vermiculite (0.7-2.0 mm), or biochar (0.3-6.0 mm) was selected as the substrate, the detailed information is shown in our previous study (Hu et al., 2022). Ibuprofen, diclofenac, hydrochlorothiazide, chloramphenicol, furosemide, gemfibrozil, triclosan, and triclocarban were selected as the representative PPCPs. Synthetic wastewater with

different concentrations of selected PPCPs was added to each CW. The composition of synthetic wastewater and concentrations of selected PPCPs are shown in our previous study (Hu et al., 2022). All chemicals and standards were analytical-grade and provided by Sigma-Aldrich (Schnelldorf, Germany).

6.3.2 Experimental design

A total of 48 mesocosms were used in this study. Each mesocosm was consisted of the following layers: a 15 cm-layer of gravel at the bottom, a 20 cm-layer of the seleced substrate in the middle, and a 15 cm-layer of sand on top. Sand (0.1-0.6 mm), expanded perlite (0.1-2.0), expanded vermiculite (0.7-2.0 mm), or biochar (0.3-6.0 mm) were individually filled in the middle layer to perform four CW systems with different substrates. For each CW system, 12 mesocosms were used (**Fig. 6.1**), envenly divided into four mesocosm treatments in triplicates based on plant presense (planted with or without *G. maxima*) and AMF inoculation (AMF- or AMF+ treatments). In AMF+ treatments, 350 g fungal inoculum was added in the middle layer, while in AMF- treatments, equivalent amounts of sterilized fungal inoculum and 100 mL inoculum filtrate (20 μm pore size) were added in the middle layer. Full details of the experimental setup are described in our previous study (Hu et al., 2022).

Due to the positive effects of fluctuating water regimes on AMF colonization (Hu et al., 2020b), CWs were operated with a flood-drain water condition during the whole experiment. The frequency of flood-drain water condition is 2 L/4 d, providing a two-hour “flood” phase and a ninety-four-hour “drain” phase. In the first two months, CWs were only fed with 10% strength synthetic wastewater to maintain the growth of plants and the development of symbiosis between AMF and plant roots. This study was carried out in a natural environment with rain protection from June to November.

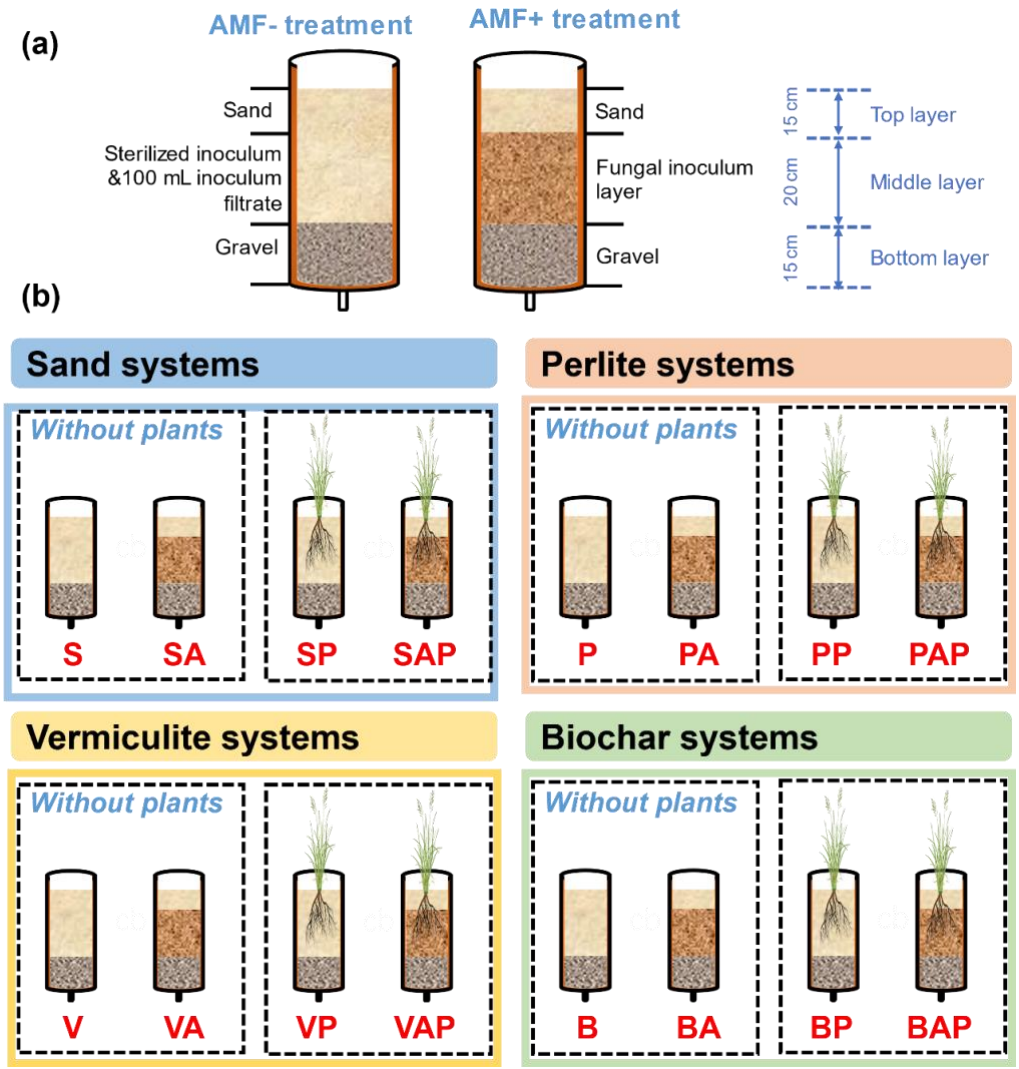


Fig. 6.1. (a). The composition of CW systems with AMF- and AMF+ treatments. (b). Schematic diagram of CW systems with different substrates in this study. S, P, V, and B represent different substrate systems filled with sand, perlite, vermiculite, and biochar systems, respectively; SA, PA, VA, and BA represent different substrate systems under AMF treatments. SP, PP, VP, and BP represent different substrate systems planted with *G. maxima*. SAP, PAP, VAP, and BAP represented different substrate systems under AMF treatments and were planted with *G. maxima*. Each treatment has three replicates. A total of 48 mesocosm-scale CWs were used in this experiment.

6.3.3 Sample analysis

In order to investigate the effects of AMF on the performance of CW for wastewater purification, the influent and effluent from each system were collected regularly every eight days. Wastewater parameters (total organic carbon (TOC), total nitrogen (TN), PO_4^{3-}-P, NO_3^--N, NO_2^--N, and NH_4^+-N) and physicochemical parameters (pH, dissolved oxygen (DO), oxidation-reduction potential (ORP), and electrical conductance (EC)) of each system were measured according to the methods as described in our previous study (Hu et al., 2021a).

6.3.4 Data analysis

The removal efficiency of conventional wastewater parameters was calculated by mass balance as described in our previous study (Hu et al., 2021a). Data are presented as mean and standard errors from the three replicates in each treatment. The student's *t*-test was used to compare the effects of AMF on plant growth; $p < 0.05$ was set as a significant difference. Plant growth status, conventional wastewater parameters, and the physicochemical parameters of CWs were analyzed using a two-way analysis of variance (ANOVA) with AMF and substrate as the primary factors and AMF*Substrate as the interaction effect. The results of physicochemical parameters (DO, EC, pH, and ORP) and conventional wastewater parameters (TOC, PO_4^{3-}-P, NH_4^+-P, TN, NO_3^--N, and NO_2^--N) were applied to principal component analysis (PCA) to examine the impacts of AMF and substrate on the performance of CWs. Statistical analyses and data visualization was achieved by R software (Version 4.0.5).

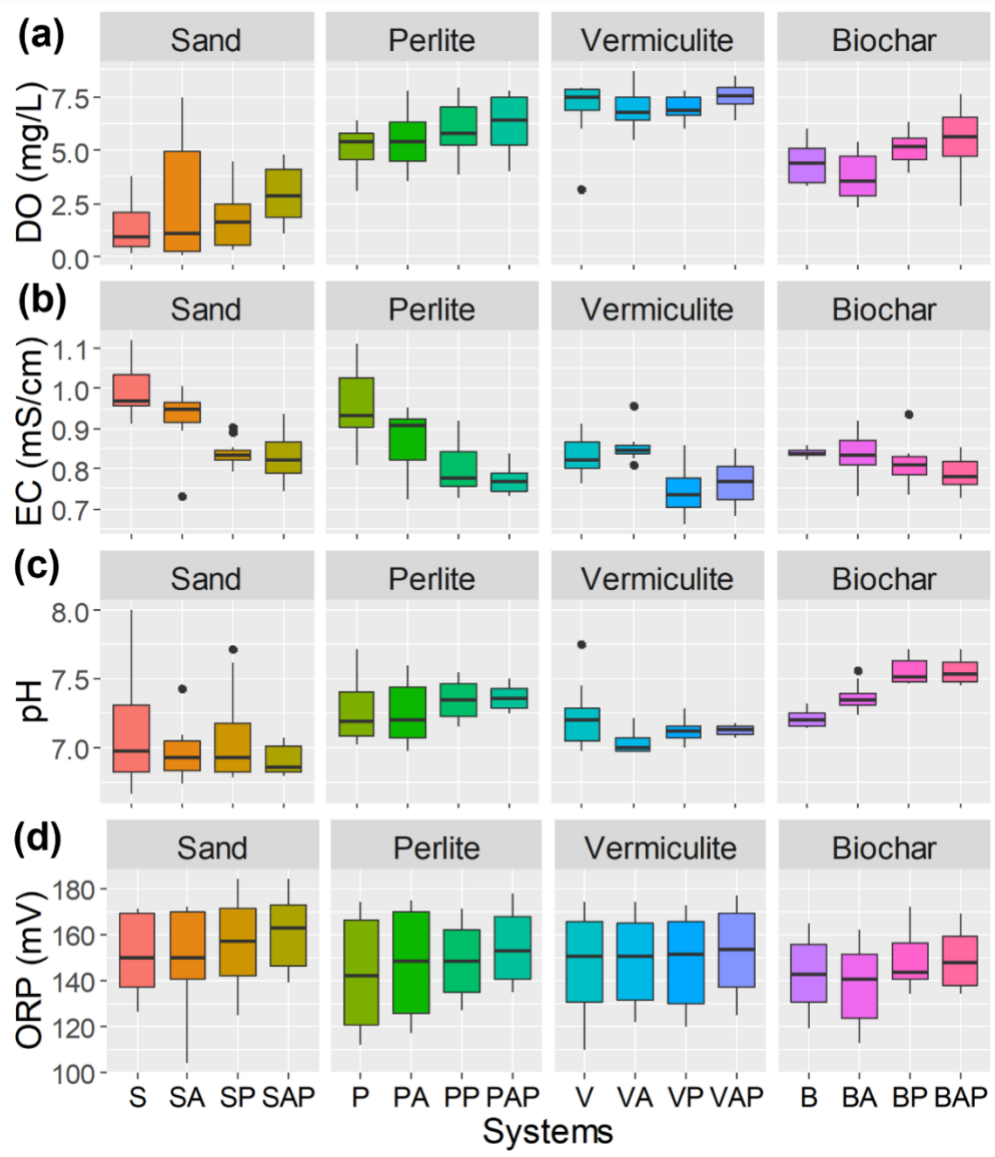


Fig. 6.2. The physiochemical parameters of CW systems. (a). DO; (b). EC; (c). pH; (d). ORP. S, P, V, and B represent different substrate systems filled with sand, perlite, vermiculite, and biochar systems, respectively; SA, PA, VA, and BA represent different substrate systems under AMF treatments. SP, PP, VP, and BP represent different substrate systems planted with *G. maxima*. SAP, PAP, VAP, and BAP represented different substrate systems under AMF treatments and were planted with *G. maxima*.

6.4 Results

6.4.1 Physiochemical parameters in the CWs

The physiochemical parameters including DO, EC, pH, and ORP in different CW systems are shown in **Fig. 6.2**. The average concentration of DO in sand (2.3 mg/L) systems was lower than that in perlite (5.7 mg/L), vermiculite (7.1 mg/L), and biochar (4.7 mg/L) systems. Meanwhile, the concentrations of DO in the sand, perlite, vermiculite, and biochar systems under planted treatments were higher than those under unplanted treatments, with an increase of 4.5-21.3%, 14.8-15.5%, -0.2-9.3%, and 15.8-45.9%, respectively. Conversely, the value of EC in the sand, perlite, vermiculite, and biochar systems under planted treatments were 10.8-15.2%, 11.2-16.9%, 9.6-10.6%, and 3.9-6.0% lower than that under unplanted treatments, respectively. Compared with unplanted treatments, the pH value in planted treatments was higher in perlite and biochar systems but lower in sand and vermiculite systems. The addition of adsorptive substrate enhanced DO and pH but decreased EC compared with sand systems under the same treatments. Meanwhile, according to the results of two-way ANOVA (**Table 6.1**), substrate had significant effects on DO, EC, and pH in CWs under both plant treatments (planted or unplanted) ($p < 0.01$), while AMF showed significant effects on DO in CWs under planted treatments ($p < 0.01$) but insignificant effect on physicochemical parameters in CWs under unplanted treatments ($p > 0.05$).

6.4.2 Wastewater parameters

TOC removal

The removal efficiency of TOC in different CWs is shown in **Fig. 6.3a**. TOC removal in CWs with the different substrates was in the following order: sand < perlite < vermiculite < biochar. The removal efficiencies of TOC in adsorptive substrate systems were 3.8-7.12% and 5.7-11.4% higher than that in sand systems with both non-planted and planted treatments, respectively. Meanwhile, AMF showed more positive

effects on TOC removal in planted CWs (1.4-3.0%) than non-planted CWs (0.1-1.0%). The results of two-way ANOVA indicated that substrate had significant effects on TOC removal in CWs ($p < 0.01$), and AMF showed significant impacts on TOC removal in planted CWs ($p < 0.01$) (**Table 6.1**).

Table 6.1. Two-way ANOVA on conventional wastewater parameters in CW systems. AMF and Substrates as main factors and AMF *Substrates as an interaction effect. *: $p < 0.05$; **: $p < 0.01$.

Index	Unplanted treatments			Planted treatments		
	AMF	Substrates	Substrates*AMF	AMF	Substrates	AMF *Substrates
DO	0.412	**	0.144	**	**	0.556
EC	0.051	**	*	0.315	**	0.629
pH	0.283	**	0.055	0.314	**	0.154
ORP	0.936	0.46	0.882	0.205	0.196	0.944
TOC	0.280	**	0.869	**	**	0.586
PO_4^{3-}-P	0.425	**	*	**	**	0.077
TN	0.070	**	0.975	0.286	**	0.433
NH_4^+-N	0.104	**	*	*	**	*
NO_3^--N	0.502	**	0.938	**	**	*
NO_2^--N	0.054	*	0.092	**	**	**

PO_4^{3-}-P removal

The removal efficiency of PO_4^{3-}-P in different CWs is shown in **Fig. 6.3b**. The removal efficiencies of PO_4^{3-}-P in CWs with different substrates were in the following order: sand < perlite < vermiculite < biochar. PO_4^{3-}-P removal in adsorptive substrate systems was 11.6-30.6% and 14.2-23.4% higher than sand systems with non-planted and planted treatments. *G. maxima* significantly enhanced PO_4^{3-}-P removal, which the removal efficiencies of PO_4^{3-}-P in planted CWs were 9.2-16.4% and 7.5-20.4% higher than that in non-planted CWs with AMF- and AMF+ treatments, respectively. Compared with AMF- treatments, the removal efficiencies of PO_4^{3-}-P under AMF+ treatments were increased (0.2-5.0%) in planted CWs but decreased in non-planted CWs. The results of two-way ANOVA indicated that substrate had significant effects

on PO_4^{3-}-P removal in CWs ($p < 0.01$) and AMF showed significant effects on PO_4^{3-}-P removal in planted CWs ($p < 0.01$) (**Table 6.1**).

Nitrogen removal

NH_4^+-N removal in the sand system was 66.5-82.4%, while the removal efficiencies of NH_4^+-N in perlite, vermiculite, and biochar systems were 16.5-31.2% higher than that in the sand system (**Fig. 6.3c**). The removal efficiencies of NH_4^+-N in CWs with different substrates were in the following order: sand < biochar < perlite vermiculite. Meanwhile, the presence of plants showed positive effects on NH_4^+-N removal, and the removal efficiencies of NH_4^+-N in planted systems were 0.5-15.0% and 0.8-15.7% higher than that in non-planted CWs with both AMF- and AMF+ treatments, respectively. In addition, AMF played more important roles in removing NH_4^+-N in sand systems than others. The removal efficiency of NH_4^+-N in planted sand systems under AMF+ treatments was 6.7% higher than that under AMF- treatments, while insignificant effects of AMF on NH_4^+-N removal were observed in other substrate systems.

The removal efficiency of TN in different CWs is shown in **Fig. 6.3d**. The removal efficiencies of TN in CWs with different substrates were in the following order: sand < perlite < vermiculite < biochar. TN removal in adsorptive substrate systems was 6.2-34.6% and 14.1-22.7% higher than that in sand systems with non-planted and planted treatments, respectively. Meanwhile, the removal efficiencies of TN in planted CWs were 11.8-32.5% and 7.5-33.4% higher than that in non-planted CWs with AMF- and AMF+ treatments, respectively. Compared with AMF- treatments, the removal efficiencies of TN in CWs with AMF+ treatments were increased 2.4-4.6% and 0.2-3.6% in both planted and non-planted treatments, respectively.

The results of two-way ANOVA indicated that substrate had significant effects on NH_4^+-N and TN in CWs ($p < 0.01$), and AMF showed a significant impact on NH_4^+-N removal ($p < 0.05$) but insignificant effects on TN removal ($p > 0.05$) in planted CWs (**Table 6.1**).

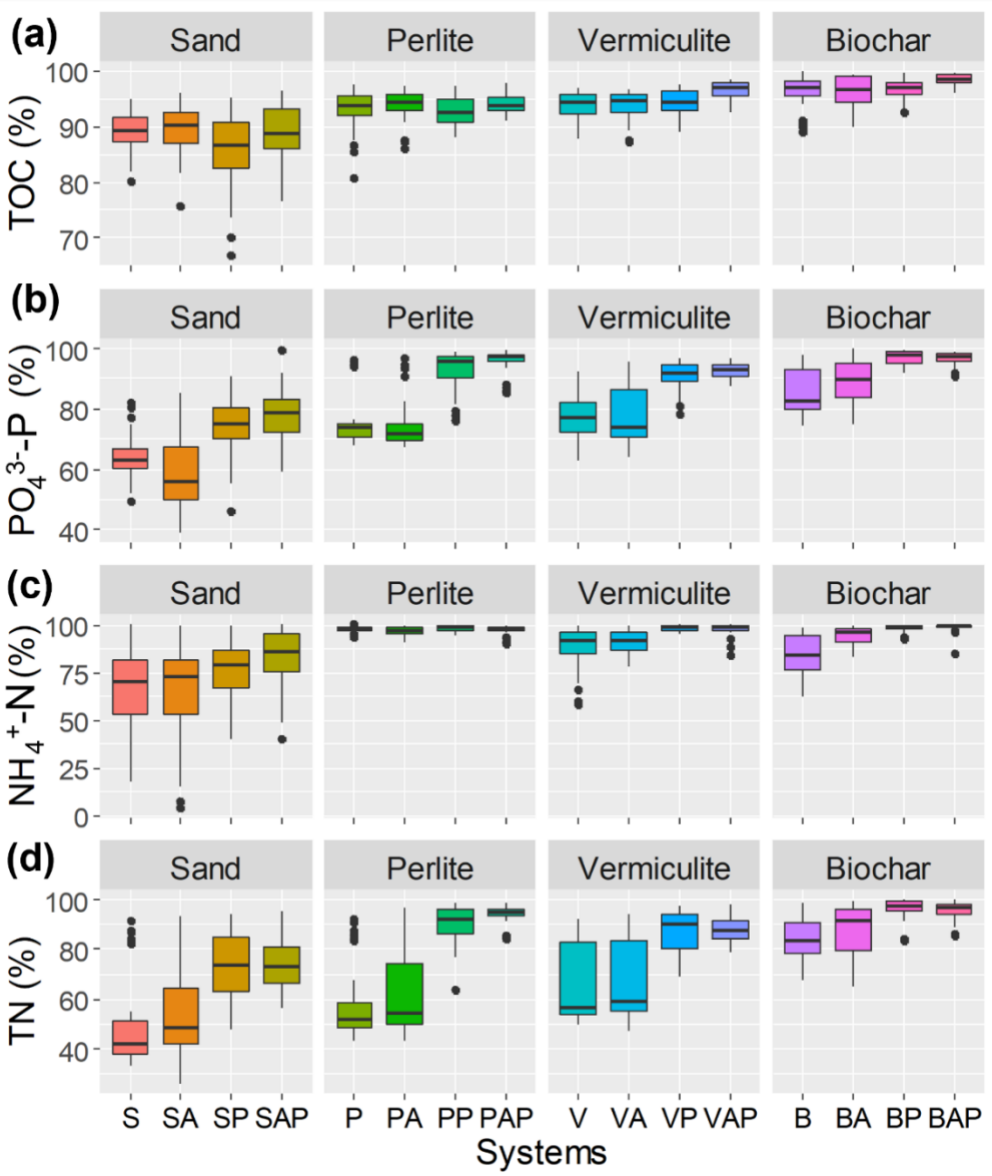


Fig. 6.3. The removal efficiency of conventional wastewater parameters in CW systems. (a). TOC; (b). PO_4^{3-}-P; (c). NH_4^+-P; (d). TN. S, P, V, and B represent different substrate systems filled with sand, perlite, vermiculite, and biochar systems, respectively; SA, PA, VA, and BA represent different substrate systems under AMF treatments. SP, PP, VP, and BP represent different substrate systems planted with *G. maxima*. SAP, PAP, VAP, and BAP represented different substrate systems under AMF treatments and were planted with *G. maxima*.

The composition of nitrogen sources in the influent were 20.9% for NH_4^+-N, 29.0% for NO_3^--N, 0.16% for NO_2^--N, and 49.9% for other nitrogen sources (**SI, Fig. S6.2**). In the effluent of all substrate systems, a significant decline was found for the composition of NH_4^+-N (0.3-5.8%) and other nitrogen sources (20.2-35.5%), whereas the composition of NO_3^--N rised up to 48.2-76.6% (**Fig. 6.4**). Moreover, the composition of other nitrogen source in non-planted CWs was 23.6-36.7% and about 3.4-7.5% lower than that in planted CWs. On the contrary, the composition of NO_3^--N in planted CWs was 59.8-74.8% and increased by 6.3-8.4% than that in non-planted CWs. However, the differences in nitrogen source utilization showed insignificant between AMF- and AMF+ treatments in both planted and unplanted systems.

6.4.3 Correlation between physicochemical parameters and conventional wastewater parameters

As shown in **Fig. 6.5**, the relations between physicochemical parameters and conventional wastewaters were significantly different among the four substrates CWs. In sand systems, the first two components of PCA explained 71.6% of the total data variability; the first principal component (Dim1) had negative correlations with ORP and TOC but positively related to NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P, TN, and EC (**Fig. 6.5a**). In perlite systems, Dim1 explained 75.5% of the variability, which had high positive correlations ($r > 0.8$) for TOC, ORP, DO, and pH and negative correlations ($r < -0.8$) for TN, NO_3^--N, PO_4^{3-}-P and EC (**Fig. 6.5b**). In vermiculite systems, Dim1 accounted for 53.9% of the variance and had positive correlations ($r > 0.8$) for NO_3^--N, TN, NO_2^--N, P, and EC, while the second principal component (Dim2) explained 20.1% of the variability and showed positive correlations ($r > 0.8$) for DO and ORP (**Fig. 6.5c**). In biochar systems, Dim1 and Dim2 represent 57.5% and 20.2% variability, respectively; TN, PO_4^{3-}-P, NH_4^+-N, NO_2^--N and EC were highly dependent upon each other ($r > 0.75$), while DO, ORP, pH, and TOC had negative correlations ($r > 0.6$) on Dim1(**Fig. 6.5d**). In addition, the PCA score of each system is shown in **Fig. 6.6**. A clear distinction can be found between planted and unplanted treatments, based on separation along Dim1. Meanwhile, the difference between AMF- and AMF+ treatments also can be found in planted CWs but showed insignificant in non-planted CWs.

6.5 Discussion

6.5.1 Effects of vegetation and substrate on pollutant removal

In this study, vegetation (*G. maxima*) plays a vital role in wastewater purification compared to the unplanted CWs. Similar results were reported by Chand et al. (2021) who found the removal efficiency of pollutants (e.g., NH_4^+-N, P, and sulfate) from wastewater in planted (*Typha* sp.) CWs were higher than that in unplanted treatments. Wetland plants can play an essential role in removing contaminants in CWs, which can assimilate pollutants directly into their tissues and enhance the environmental diversity in the rhizosphere to promote a variety of physical, chemical, and biological reactions for contaminant removal (Hu et al., 2021c). The PCA analyses also confirmed that the physicochemical and conventional wastewater parameters showed significant differences between planted and unplanted CWs (**Fig. 6.6**). Dordio and Carvlho (2013) reported that plant presence facilitated the release of oxygen, thus providing favorable conditions for the growth of microorganisms and promoting the aerobic biodegradation of pollutants in CWs. In this study, DO concentrations in planted CWs were higher than in unplanted systems (**Fig. 6.2**). Since the concentration of TP in the aqueous phase is negatively correlated with oxygen levels (Vohla et al., 2007), the enhancement of oxygen condition in planted CWs may also contribute to P removal. Meanwhile, the presence of *G. maxima* increased the DO concentration, which may also promote the nitrification process (Fu et al., 2020). In addition, plant roots can provide direct attachment sites for microbes and suitable environments for microbial development and metabolism in the rhizosphere (A. et al., 2020; Zhang et al., 2022), thus enhancing the microbial degradation of pollutants in CWs.

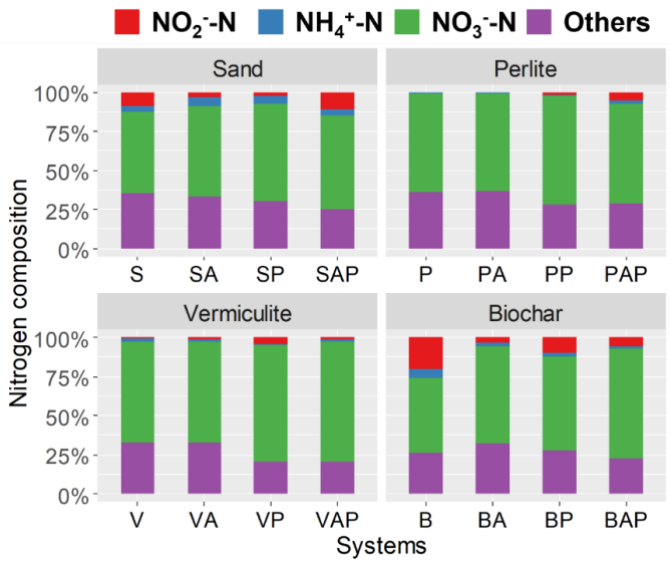


Fig. 6.4. The nitrogen composition in the effluent of CW systems. S, P, V, and B represent different substrate systems filled with sand, perlite, vermiculite, and biochar systems, respectively; SA, PA, VA, and BA represent different substrate systems under AMF treatments. SP, PP, VP, and BP represent different substrate systems planted with *G. maxima*. SAP, PAP, VAP, and BAP represented different substrate systems under AMF treatments and were planted with *G. maxima*.

CWs filled with the adsorptive substrate (perlite, vermiculite, and biochar) have a better performance for wastewater purification. Compared with sand systems, the removal efficiencies of TOC, NH_4^+-N, TN, and PO_4^{3-}-P in adsorptive substrate systems with both planted and unplanted treatments were increased by 3.8-11.4%, 16.5-31.4%, 6.2-34.6%, and 11.6-30.6%, respectively (**Fig. 6.3**). The substrate selection significantly affects pollutant removal in CWs (Wang et al., 2020). This result also accords with our recent observations, indicating that the application of perlite, vermiculite, and biochar in CWs had a better removal performance for PPCPs than sand (Hu et al., 2022). The main reason could be that perlite, vermiculite, and biochar have a higher sorption capacity for contaminants than sand (Thomaidi et al., 2022; Yang et al., 2018), which could enhance the retention of pollutants in CWs. As shown in **SI, Fig. S6.1**, the surface morphology characterization of substrate indicated that the surface of perlite, vermiculite, and biochar are rough, porous, and multi-cracked, while there are few cracks on the relatively smooth surface of quartz sand. The substrate with higher pore volume or specific surface area generally has higher adsorption and

removal performance for pollutants in CWs (Yang et al., 2018). Besides, the porous structure of substrate could be considered a perfect bio-carrier for the development of microorganisms in CWs, which benefits the formation of biofilms and the composition of microbial communities, thus enhancing the bioaccumulation and biodegradation of pollutants (Fu et al., 2020). Liu et al. (2014) also demonstrated that the rapid and stable removal of pollutants in zeolite-based tidal flow CWs could be attributed not only to the high adsorption capacity of the specific substrate during the flooded phase but also to the fast biodegradation during the "drained" phase of each tidal operation.

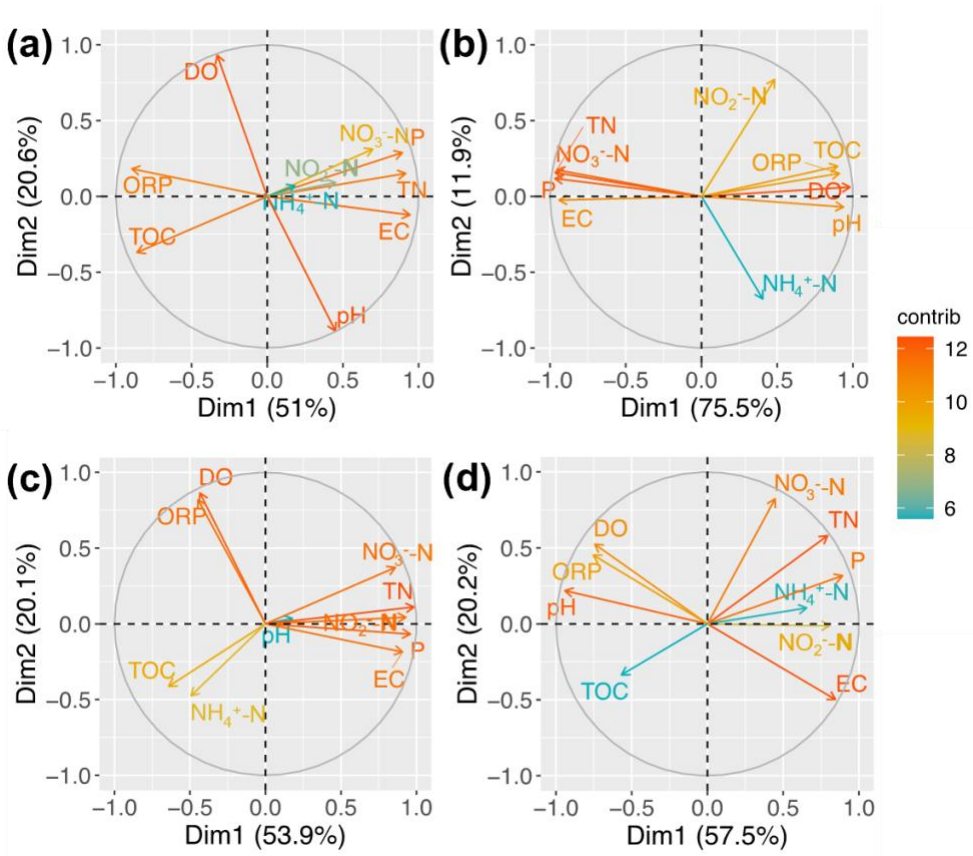


Fig. 6.5. Principle component analysis (PCA) among physicochemical parameters (DO, EC, pH, and ORP) and conventional wastewater parameters (TOC, PO_4^{3-}-P, NH_4^+-P, TN, NO_3^--N, and NO_2^--N) in CW systems. (a) Sand systems; (b) Perlite systems (c) Vermiculite systems; (d) Biochar systems.

In addition, the differences in physicochemical parameters among the four substrate systems may have a significant impact on pollutant removal (**Fig. 6.2**). It was confirmed by the PCA analyses, indicating that the correlation between physicochemical parameters and conventional wastewater parameters were significantly different among the four substrate systems (**Fig. 6.5**). Previous studies proved that the physiochemical parameters (e.g., DO, pH, ORP, and EC) could directly influence microbial abundance and species (Fu et al., 2020; Li et al., 2014), thus affecting the ultimate performance of wastewater purification. In this study, the average concentrations of DO in the effluent of perlite, vermiculite, and biochar systems with unplanted treatments were significantly higher than those of sand systems under the same conditions (**Fig. 6.2a**). Consequently, there was a better aerobic condition in perlite, vermiculite, and biochar systems for nitrification, aerobic denitrification, and biodegradation. Meanwhile, the higher dissolved oxygen may also contribute to the accumulation of phosphorus inside CW systems (Vohla et al., 2007). These results are in accordance with findings reported by Bai et al. (2021), who observed that the porous substrate could enhance the transport of oxygen, which is conducive to the growth, propagation, and biochemical processes of aerobic microorganisms, resulting in bioaccumulation and biodegradation of more organic compounds (Ji et al., 2022). Fu et al. (2020) also demonstrated that using substrates with porous structures such as activated carbon could improve the dissolved oxygen supply and enhance the removal efficiency of pollutants in CWs.

6.5.2 Effects of AMF on contaminates purification in CWs

AMF inoculation significantly enhanced the removal of TOC, NH_4^+-N, and P from wastewater in planted CWs, indicating that AMF might have an essential role in wastewater purification in CWs. The reason could be that AMF promoted plant growth and reduced the adverse effects of PPCPs stress (Hu et al., 2021a). In our study, the root length, shoot height, and biomass of AMF inoculated *G. maxima* were increased compared to the AMF- treatments (Hu et al., 2021d), which might provide more sites for the development of microorganisms and a high oxygen release rate, thereby beneficial for the biodegradation of the pollutants in CWs (Keerthanan et al., 2021). Similar results also suggested that biofilters planted with inoculated *Ficinia nodosa* had

10% higher removal of TN and 5% higher removal of TP than the non-inoculated controls (Palacios et al., 2021). In addition, the extraradical hyphae of AMF might also contribute to pollutant removal by facilitating efficient uptake of immobile nutrients by the host plant (Ajit Varma, 2017; Huang et al., 2021). By using isotopic labeling techniques, previous studies identified direct evidence that the extraradical hyphae of AMF could provide the mycorrhizal pathway for the acquisition of P and N (Mäder et al., 2000; Püschel et al., 2021), resulting in a more efficiently accumulation of P and N in inoculated plants than that in non-inoculated plants. Moreover, AMF might also influence pollutant removal by modifying root exudates to stimulate microbial activity, biochemical transformations, and enhancement of mineralization in the rhizosphere (Monther and Kamaruzaman, 2012). Corgié et al. (2006) investigated the contribution of AMF (*Glomus mosseae*) to phenanthrene biodegradation in the presence of ryegrass (*Lolium perenne* L.), suggesting that AMF increased the density of culturable heterotrophic and phenanthrene degrading bacteria and the activity of dioxygenase transcription in the rhizosphere.

6.5.3 Effects of interactions between substrates and AMF on wastewater purification in CWs.

Substrates may also influence the functional role of AMF in pollutant removal in CWs. The results of Two-way ANOVA indicated that AMF*substrate showed significant effects on nitrogen forms (e.g., NH_4^+-N, NO_3^--N, and NO_2^--N) in CWs (**Table 6.1**). The possible reason is that substrate types could affect the development of AMF symbiosis, which indirectly affects the growth of host plants, resulting in the variability of pollution removal in CWs with a different substrate. Our previous study investigated AMF colonization in planted CWs with a different substrate, confirming that mycorrhizal status in the roots of *G. maxima*, including mycorrhizal frequency, mycorrhizal colonization, and arbuscule abundance, showed significant differences among sand, perlite, vermiculite, and biochar systems (Hu et al., 2021d). It is worth noting that the effects of AMF on NH_4^+-N removal were significant in planted sand systems but insignificant in planted adsorptive substrate systems (**Fig. 6.3**). The possible explanation is that the “drain” phase was only two hours per cycle in the present study. Pollutants were challenging to be quickly eliminated from the aqueous phase by biodegradation or plant uptake due to the short residence time of wastewater (Jia et al., 2010). Consequently, the adsorption of substrates and the attached microbes

could play the dominant roles in pollutant removal in the present study. The effects of AMF on promoting pollutants removal in sand systems could be more relatively significant than adsorptive substrate systems. By contrast, the porous structure of perlite, vermiculite, and biochar in flood-drain CWs can provide a more critical role in pollutant removal (**Fig. 6.3**) and plant growth (Hu et al., 2021d) than AM symbiosis. These were confirmed by the results of Two-way ANOVA, indicating that substrate showed more significant effects on physicochemical parameters and pollutant removal than AMF in tidal flow CWs (**Table 6.1**).

6.6 Conclusion

Adsorptive substrates (perlite, vermiculite, and biochar), AMF, and *G. maxima* significantly increased the pollutants (TOC, TN, PO_4^{3-}-P, and NH_4^+-N) removal from wastewater in CWs. The application of adsorptive substrates in CWs showed a more critical role in pollutant removal than AMF inoculation. These results suggested that adsorptive substrate addition and plants presence could improve the performance of CWs to purify wastewater, and the symbiotic relationship between AMF and plant roots might have the potential to enhance pollutant removal in CWs. Based on this study, further studies are needed to reveal the impacts of AMF on pollutant metabolic pathways in CWs, including microbial degradation, plant uptake, and substrate adsorption. To approach the need of actual engineering applications, besides, future studies should be conducted to assess the impacts of AMF on pollutant removal in more realistic conditions, such as full-scale CW systems, real wastewater, and more suitable hydraulic conditions (e.g., a long-term flooding condition or a tidal flow with more frequent water change cycle).

6.7 Supplementary Materials

Figures:

- Fig. S6.1. The characteristics of substrates. SEM images and EDX spectra of sand, vermiculite, perlite, and biochar
- Fig. S6.2. The composition of nitrogen sources in the influent of CW systems

Figures:

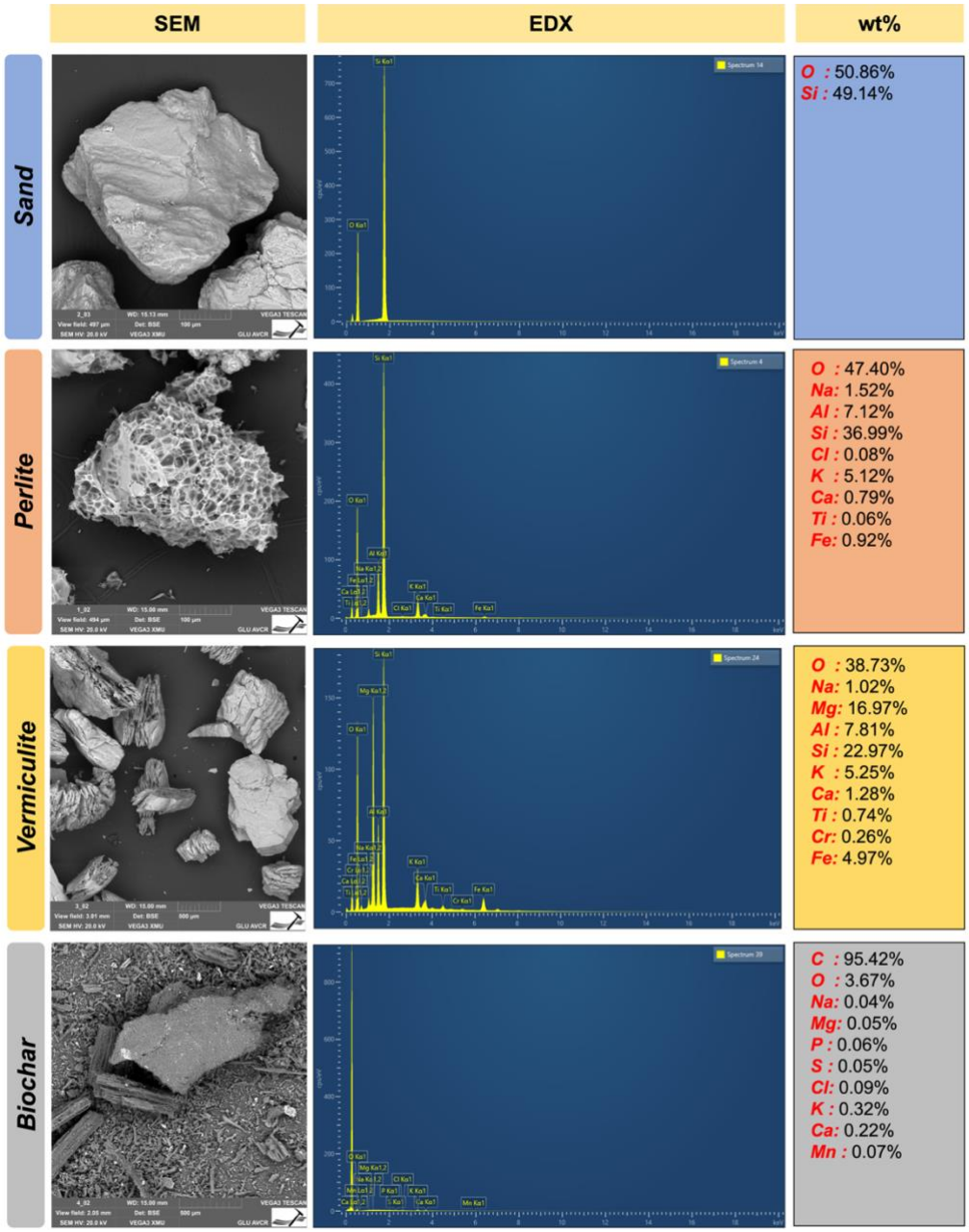

SEM
EDX
wt%
Sand
O : 50.86%
Si : 49.14%
Perlite
O : 47.40%
Na: 1.52%
Al : 7.12%
Si : 36.99%
Cl : 0.08%
K : 5.12%
Ca: 0.79%
Ti : 0.06%
Fe: 0.92%
Vermiculite
O : 38.73%
Na: 1.02%
Mg: 16.97%
Al : 7.81%
Si : 22.97%
K : 5.25%
Ca: 1.28%
Ti : 0.74%
Cr: 0.26%
Fe: 4.97%
Biochar
C : 95.42%
O : 3.67%
Na: 0.04%
Mg: 0.05%
P : 0.06%
S : 0.05%
Cl: 0.09%
K : 0.32%
Ca: 0.22%
Mn : 0.07%

Fig. S6.1. The characteristics of substrates. SEM images and EDX spectra of sand, vermiculite, perlite, and biochar.

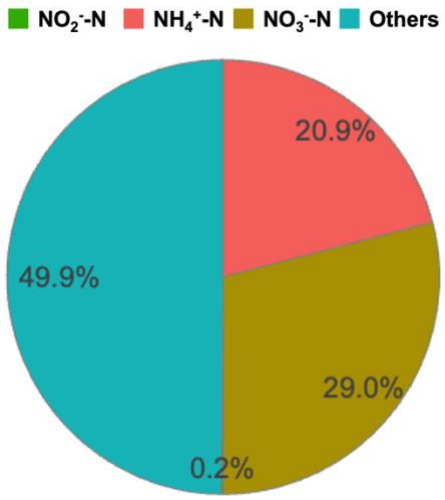


Fig. S6.2. The composition of nitrogen sources in the influent of CW systems.

Chapter VII

Summary

The symbiotic relationships between AMF (*R. irregularis*) and plant roots (*G. maxima*) could successfully establish in CWs. However, mycorrhizal status in plant roots showed differences among the four substrate systems. The addition of adsorptive substrates (perlite, vermiculite, or biochar) in CWs was more conducive to forming AM symbiosis than sand. AMF enhanced nutrient uptake by the host plant, thereby significantly promoting the growth of *G. maxima*. Meanwhile, AM symbiosis had an essential function in CWs, which improved host plant resistance to EOPs' stresses by increasing antioxidant enzymes (POD and SOD) and soluble protein contents in plant tissues and decreasing oxidative damage (MDA and $O_2^{\bullet-}$).

The transformation of EOPs and their metabolites in plant tissues differed among the eight selected EOPs. Ibuprofen, diclofenac, hydrochlorothiazide, chloramphenicol, furosemide, gemfibrozil, and EOPs' metabolites (2-hydroxy ibuprofen, carboxy ibuprofen, and 4'-hydroxy diclofenac) mainly accumulated in the roots, but triclosan and triclocarban are more likely to accumulate in the shoots. Surprisingly, AM symbiosis enhanced the translocation of EOPs from roots to shoots in all substrate systems, resulting in a higher amount of EOPs in the shoots of inoculated plants than that of non-inoculated plants. Meanwhile, AM symbiosis promoted the accumulation of EOPs in plant roots in sand and vermiculite systems, but an opposite effect of AMF on the accumulation of EOPs in plant roots was observed in perlite and biochar systems.

The addition of adsorptive substrates (perlite, vermiculite, or biochar) promoted the removal efficiencies of EOPs in CWs because they had positive effects on enhancing the accumulation of EOPs in the rhizosphere and facilitating the uptake of EOPs by plant roots. Meanwhile, AMF might potentially influence EOPs removal in CWs, and this ability could be affected by the type of substrates. The contents of all selected EOPs (except triclosan, which the concentration was below the detection limit) and their metabolites (2-hydroxy ibuprofen and carboxy ibuprofen) in the rhizosphere soil of sand and perlite systems with AMF inoculated treatments were higher than those in non-AMF controls. However, a lower accumulation of EOPs (e.g., hydrochlorothiazide and triclocarban) was observed in the rhizosphere of vermiculite and biochar systems with AMF treatments were lower than those in non-AMF controls.

The predominant removal process of EOPs in the present study could be attributed to the adsorption of substrates and the attached microbes. Consequently, AMF had positive effects on pollutant removal in sand systems but insignificant effects in perlite, vermiculite, and biochar systems. In sand systems, AM symbiosis improved the treatment performance of conventional pollutants. Compared to non-AM inoculated

treatments, the removal efficiency of TOC, PO_4^{3-}-P, NH_4^+-N, TN in AMF inoculated treatments significantly improved by 6.19%, 10.92%, 14.82%, and 10.92%, respectively. For EOPs removal (ibuprofen and diclofenac as an example), the removal efficiencies of ibuprofen and diclofenac in sand systems with AMF inoculated treatments were 5.82-13.88% and 2.23-21.07% higher than those with non-AMF inoculated treatments, respectively. It is worth noting that the addition of AMF effectively reduced the concentration of EOPs' metabolites (2-hydroxy ibuprofen, carboxy ibuprofen, and 4'-hydroxy diclofenac) in the effluent of all four substrate systems.

Although the application of adsorptive substrates in CWs showed a more critical role in pollutant removal than AMF inoculation, AMF may also have the potential to enhance the performance of CWs for wastewater purification. Our results provide a good starting point for applying AMF in the phytoremediation of EOPs in full-scale wetland systems. Based on this study, additional studies are necessary to investigate the role of AMF in the purification of wastewater in more realistic conditions, such as treating real wastewater, feeding with more suitable hydraulic conditions (e.g., a long-term flooding condition or a tidal flow with more frequent water change cycle), or studying on large-scale systems. Meanwhile, further attention should be paid to the mechanisms by which AMF affects EOPs removal in CWs, for example, the impacts of AMF on EOPs' metabolic pathways in CWs, including microbial degradation, plant uptake, adsorption and/or desorption by substrate and attached microbes.

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Curriculum vitae

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Publications

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Chen Z., **Hu B.**, Hu S., Vogel-Mikušb, K., Pongracb P., Vymazal J., Decrease in chromium mobility in yellow iris (Iris pseudacorus L.) under semi-aquatic conditions due to the application of arbuscular mycorrhizal fungus and biochar. (**Submit to Water Research**).

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Grants and projects

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