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Enhanced nutrient removal and mechanisms study in benthic fauna added surface-flow constructed wetlands: the role of *Tubifex tubifex*

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Abstract

This study designed a combined benthic fauna-*T. Orientalis*-substrate-microbes surface-flow constructed wetlands (SFCWs) through the addition of *T. tubifex*. Results showed that, the removal efficiencies of nitrogen and phosphorus in the tested SFCWs achieved 81.14±4.16% and 70.49±7.60%, which were 22.27% and 27.35% higher than that without *T. tubifex*. Lower nitrate (2.11±0.79 mg/L) and ammonium (0.75±0.64 mg/L) were also observed in the tested SFCWs, which were 3.46 mg/L and 0.52 mg/L lower than that without *T. tubifex*. Microbial study confirmed the increased denitrifiers with *T. tubifex*. The lower nitrogen in effluent was also attributed to higher contents of nitrogen storage in sediment and *T. orientalis* due to the bioturbation of *T. tubifex*. Furthermore, with *T. tubifex*, higher proportions of particulate (22.66±3.96%) and colloidal phosphorus (20.57±3.39%) observed promoted phosphorus settlement and further absorption by *T. orientalis*. The outcomes of this study provides an ecological and economical strategy for improving the performance of SFCWs.

Keywords: Constructed wetlands; *Tubifex tubifex*; inorganic nitrogen; phosphorus transformation; microbial mechanism

1. Introduction

Constructed wetlands (CWs), known as an efficient ecological technology, artificially enhance the natural processes to alleviate excessive nutrients in aquatic ecosystems. Compared to other treatment systems, CWs are easy to operate, more environmental friendly and cost-effective. The well-known removal mechanisms of nitrogen (N) in traditional CWs are substrate adsorption, plant absorption and microbial degradation, especially the processes of nitrification and denitrification (Coban et al., 2015a; Zhi et al., 2015). Meanwhile, the mechanisms of phosphorus (P) removal in CWs are also plant uptake, microbial growth, substrate adsorption and chemical precipitation, especially substrate adsorption (Blanco et al., 2016). However, the effect of benthic fauna on CWs performance is generally ignored.

According to previous report, the bioaccumulation, bioremediation, biostabilization and biodegradation are significant for the control of pollutants (Gifford et al., 2007). Furthermore, the energy and biomass transformation by animals in trophic chains is crucial for the ultimate fate of some pollutants in aquatic environment (Ding et al., 2015). Thus, to improve the nutrient removal in CWs through combined biological, physical and chemical interactions among integrated plant-animal-substrate-microbes was highlighted. As a typical kind of meiofauna, high-density *Tubifex tubifex* (*T. tubifex*) can be found in nutrient-enriched freshwater sediment because of its high tolerance to polluted environments, even in low-oxygen conditions (Tian & Lu, 2010). As a common deposit feeder and head-down conveyor, the *T. tubifex* live submerged in sediment superficial layers, using anterior section for predation and tail of the posterior section for respiration and defecating (Tian et al., 2012). Their extensive bioturbation activity have significant influence on downward migration and porosity of freshwater sediment (Nogaro & Mermillod-Blondin, 2009).

Due to its potential role in contaminant transformation and high density in most wetland habitats, *T. tubifex* was selected in this study as a benthic fauna to enhance the performance of CWs.

In CWs without benthic faunas, the N and P removals are highlighted to be enhance. As the advanced treatment stage, the wastewater in CWs is usually the effluent from conventional biological wastewater treatment in municipal wastewater treatment plant. Thus, the feed wastewater for CWs usually has low C/N ratio (Hallin et al., 2015; Wu et al., 2013). Despite the effect of rhizosphere or periphyton of macrophyte species, the carbon source is still insufficient for denitrification, especially in surface-flow CWs (SFCWs). Thus, CWs show low efficiency in reduction of nitrate (NO_3-N) and total nitrogen (TN) (Chen et al., 2014; Zhi et al., 2015). Moreover, the main pathways of P removal in CWs by plants absorption and substrate adsorption are affected by the magnitude, particulate size and settlement activity of P, as well as the limited interaction among the sediment, plants and microbes (Blanco et al., 2016; Heathwaite & Dils, 2000; Pettersson, 2001; Wang et al., 2015c).

We hypothesized that, the performance of N and P removals in CWs may be improved by the addition of *T. tubifex*. Firstly, the bioturbation activity may affect the oxygen level in water-sediment interface and superficial sediment. The interaction between *T. tubifex* and microbes may also change C/N ratio through their effect on nitrogen degradation and carbon mineralization, thereby improving NO_3-N removal (Nascimento et al., 2012). In addition, in contrast to other studies which found higher NH_4-N in wastewater with *T. tubifex* (Hendrickx et al., 2011), we hypothesized that *T. tubifex* can promote NH_4-N removal in CWs despite its extraction. The bioturbation activity by *T. tubifex* and the ecological environment provided by CWs systems may beneficial for subsequent NH_4-N deposited and removal. Thus, *T. tubifex* could play an important role in N-transformation due to their biological effect on nitrification and denitrification processes. Secondly, two typical P forms, namely particulate and colloidal P, are especially important for P-removal. In wetlands, the particulate P can incorporate dissolved P and precipitate directly into the sediment (Noe et al., 2007). Colloidal P, suspended in wastewater, can associate with aluminum (Al), iron (Fe), calcium (Ca) and organic matter, and then bind to sediment. Furthermore, a study has proved that the uptake of P by plants was enhanced with colloidal P (Montalvo et al., 2015). Faunal activity by *T. tubifex* may have a significant effect on P-transformation due to its particulate reworking and burrowing, ventilation and bio irrigation (Stief, 2013).

Thus, an integrated *T. orientalis*-*T. tubifex*-substrate-microbes system was proposed to improve the nutrient treatment performance on CWs in this study. In this study, the dynamics of intensified N and P removal in wastewater were evaluated, and the important role of *T. tubifex* in nutrient transformation, especially NO_3-N, NH_4-N, particulate and colloidal P, was examined. The contents of inorganic N, P and organic carbon in the sediment and *T. orientalis*, as well as microbial abundance and diversity were also analyzed.

2. Materials and methods

2.1 *T. orientalis* and *T. tubifex* cultivation

Typha orientalis (*T. orientalis*), a typical wetland plant, was selected for the experiments. Sprouted *T. orientalis* of 15 to 20 cm height were transplanted into laboratory-scale continuously-operated SFCWs microcosms in March 2015 and cultivated in Hoagland solution (10%) for the first three months (Zhao et al., 2015). The planting density was 32 plants/m^2.

T. tubifex of 15 to 20 mm in length and 0.5 mm in diameter were collected from a flower-aquarium market in Jinan, Shandong Province. Before used in microcosms, the worms were cultivated in the laboratory for 15 days. In these days, the *T. tubifex* were feed with synthetic wastewater, with the DO concentration higher than 5 mg/L. After that, they were washed several times and put in the experiment units with the density ranging from 12800 to 13000 individuals/m^2, corresponding to the density range reported in nature environments (Mermillod-Blondin et al., 2013).

2.2 Laboratory-scale continuously-operated SFCWs setup

CW microcosms were established under a transparent rain shelter in School of Environmental Science and Engineering, Shandong University. Ten polyethylene barrels (38 cm in inner diameter, 45 cm in height) were used to build SFCW systems. Each microcosm was filled with two layers of substrate. The upper layer was filled with sediment, collecting from the natural wetlands of Nansi Lake, Shandong Province, China. While the bottom layer was made up of washed gravel (1-3 mm in diameter, mainly Si_2O_3, Al_2O_3, and Fe_2O_3) (Zheng et al., 2016). These SFCWs were operated in continuously-flow mode, and the inflow and outflow pre-punched tubes were positioned at the top of each barrel with the same height (Fig. S1) (Villasenor et al., 2013).

Four groups (each group has three parallels, except for the control group) were designed and operated parallelly in this study, namely 1) SFCW-A: group has both *T. orientalis* planted and *T. tubifex* addition; 2) SFCW-B: group only has *T. orientalis* planted; 3) SFCW-C: group only has *T. tubifex*; and 4) SFCW-Control: blank group only with substrate.

2.3 Experimental procedure

The experimental CW microcosms were operated for 4 months (From June 2015 to September 2015) at temperature ranging from 19 °C to 37 °C. During the experiment, each microcosm was fed with synthetic wastewater containing sucrose, $(NH_4)_2SO_4$, KH_2PO_4, KNO_3 and some micronutrients, such as Ca, Mg, S, Fe, Zn, Cu, Mn, B, and Mo. The main characteristics of the influent were as follows (mg/L): chemical oxygen demand (COD) 60.30 ± 0.37, NO_3-N 12.01 ± 0.37, NH_4-N 8.59 ± 0.30; total nitrogen (TN) 21.64 ± 0.20; and total phosphorus (TP) 0.55 ± 0.30. When filled, each system held 17 L wastewater, with a depth of 15 cm above the media. The overlying wastewater was renewed with a peristaltic pump to simulate a mode of continuously-flow CWs. The hydraulic retention time (HRT) of each SFCW was 3 days, corresponding to a hydraulic loading rate (HLR) of 5.15 cm/day.

2.4 Sampling and analysis

Water samples were collected in the overlying water at the inlet and outlet of each microcosm every three days for chemical analysis. DO and temperature were measured in situ using a dissolved oxygen meter (HQ30d 53LEDTM, HACH, USA). Parameters such as TP, TN, NH_4-N, NO_3-N, NO_2-N and COD were determined in the laboratory according to the methods described in APHA-AWWA-WPCF (2001). Dissolved P was determined after filtering using 0.45-μm cellulose acetate membrane syringe filter by applying manual pressure (Shand et al., 2000). Particulate P was calculated as a difference between TP and dissolved P. Then, 15 mL samples were filtered through a 3 kDa (~2 nm pore size) regenerated cellulose membrane centrifugal filter (Millipore 50 mL, American) via centrifugation at 4000 rpm for 20 min to determine the concentration of colloidal P (Montalvo et al., 2015).

Sediment samples were collected every 20 days for N, P, sediment organic carbon (SOC) and microbial analysis. To obtain a representative sample for each system, sediment samples were taken from the superficial depth at the same height, and five individual samples of equal amounts were homogenized. After collection, the collected sediment was sieved using the wet sieving method through a 1-mm and 40-mm mesh to remove dead plant tissues, gravel, large solid organisms and existed *T. tubifex*. Then, the sediment samples were dried at -60 °C using a freeze dryer (Unicryo MC 2 L freeze dryer, Germany) for 36 h and then sieved (0.2 mm) and stored at -20 °C for further analyses. The contents of N, P, and SOC in the sediment were measured using the methods described in supplementary materials.

The plant samples were harvested at the end of the experiment. Because of the different activities in absorption, the above and belowground fractions were collected separately. After being dried in an oven at 65 ^{o}C for 72 h and grounded to 0.149 mm, the mass fractions of C, N and P stored in the belowground and aboveground fractions of plants were measured by the thermal conductivity detector (TCD) and the elemental analyzer (vario Macro cube elemental analyzer, Germany) in the Shandong Academy of Sciences. The samples were burned at 1150 ^{o}C. Then, the products of oxynitride, oxycarbide, and others were measured by thermal conductance and infrared analysis (GB/t 30733-2014) (Sun et al., 2015).

2.5 DNA extraction, quantitative polymerase chain reaction, and high-throughput sequencing analyses

DNA was extracted from the collected sediment using a MOBIO PowerSand^{TM} DNA Isolation Kit. The absolute abundances of bacterial 16S rRNA, relative abundance of nitrifying bacteria, as well as denitrifying bacteria were quantified by quantitative polymerase chain reaction (qPCR), and the detailed information was shown in the supplementary materials. Primers of target genes used in qPCR analysis was given in Table S1.

To obtain the microbial community for each microcosm, the Illumina high-throughput sequencing was performed at the Yuanxu Biotechnology Company (Shanghai, China). The sequences shorter than 250 base pairs (bp) in length and with a quality score lower than 30 were removed from the pyrosequencing-derived data sets. Clustering of the sequencing into operational taxonomic units (OTUs) was performed via UCLUST software at 97% sequence identity (Edgar, 2010). The Simpson diversity index, Chao richness estimations and Good's coverage were calculated by Mothur analysis (http://www.mothur.org) at 3% distance level.

2.6 Statistic analysis

All measurements were performed in triplicate for each group with the results expressed as mean ± standard deviation. The program SPSS 19.0 (SPSS, Chicago, USA) was used to perform all the statistical analyses, and the variance (ANOVA) was used to test the significance of the results. The results were considered to be statistically significant when $p < 0.05$. Standard deviations of three replicates were indicated by error bars. Invisible error bars indicated that the standard deviations were smaller than the marker size.

3. Results and discussion

3.1 Nitrogen removal performance in SFCWs

The inflow and outflow concentrations of TN in wastewater for each microcosm are illustrated in Fig. 1a. In the tested microcosms, *T. tubifex* promoted N-removal in wastewater. SFCW-A achieved the highest TN removal efficiency, followed by SFCW-C, SFCW-B and SFCW-Control. With the effluent concentrations less than 6 mg/L and 8mg/L, the TN removal efficiencies in SFCW-A and SFCW-C were 81.14±4.16% and 69.42±4.34%. The TN removal efficiency in SFCW-B was 58.87±3.88%, which were 22.27% and 10.55% lower than that in SFCW-A and SFCW-C, respectively. And SFCW-Control has the TN removal efficiency of 47.90±2.96%. The reduced N in SFCW-A was mainly attributed to ingestion and new biomass formation of *T. tubifex*, sediment adsorption and plant absorption, as well as microbial degradation such as nitrification and denitrification processes.

As an important factor for N-purification and degradation of organic carbon, the DO concentration at the water-sediment interface for each group was investigated. The photosynthesis and radial oxygen loss (ROL) by *T. orientalis* provided O_2 in the CW microcosms. Results showed that SFCW-B (5.37±0.12 mg/L) had higher DO concentration at the water-sediment interface than SFCW-A (4.02±0.44 mg/L). Same tendency was also detected in SFCW-Control (3.00±0.03 mg/L) and SFCW-C (2.77±0.02 mg/L), indicating lower DO concentration at water-sediment interface with *T. tubifex*. The respiration, digestion, metabolism and bioturbation activity of *T. tubifex* increased O_2 consumption. The lower DO concentration with *T. tubifex* was beneficial for denitrification, NO_3-N reduction and thus inorganic N removal.

To further investigate the biological N-removal performance, different forms of inorganic N were tested. As shown in Fig. 1c, obvious NO_3-N removals were observed with *T. tubifex* for both SFCW-A and SFCW-C compared with SFCW-B and SFCW-Control. NO_3-N concentrations in the SFCW-A and SFCW-C effluent were

2.12±0.79 mg/L and 3.19±0.85 mg/L, with the average removal efficiency up to 82.57±6.80% and 73.80±7.51%. Compared with the removal efficiencies of 54.18±15.01% and 37.50±9.45 for SFCW-B and SFCW-Control, *T. tubifex* could significantly enhance NO_3-N removal in wastewater. The following three reasons contributed to the enhanced NO_3-N treatment by *T. tubifex*. Firstly, the lower DO concentrations at the water-sediment interface of the *T. tubifex* added SFCWs were beneficial for biological denitrification and NO_3-N removal. Secondly, as a critical factor for NO_3-N removal, the average effluent concentrations of COD for SFCW-A and SFCW-C were 24.67±3.77 mg/L and 28.34±2.18 mg/L, with the removal efficiencies of 60.61±6.11% and 54.06±3.17%, respectively. And SFCW-B has the COD effluent of 22.90±3.63 mg/L, with the removal efficiency of 62.86±6.82% ($p > 0.05$, Fig. 1b). Despite there was no significant difference in COD removal between these groups, the metabolism and bioturbation of *T. tubifex* could generally affect the degradation of organic carbon and increase the C/N ratio. Thus, *T. tubifex* could supply a sufficient and economical carbon source for denitrification in CWs. Thirdly, the inorganic fraction of N, could be ingested and passed through the gut of *T. tubifex* when water-soaked food particles were ingested, which means the denitrification and contaminant transformations could occur in the gut of *T. tubifex* (Bonaglia et al., 2014; Stief, 2013). Although the nitrification process is limited in lower DO level, there was no obvious difference in the NH_4-N effluent concentration between each group, as shown in Fig. 1d ($p > 0.05$). In the tested microcosms, the removal efficiencies of NH_4-N in the wastewater were 90.91±7.83%, 84.61±7.69%, 79.70±7.91% and 70.63±5.00% for SFCW-A, SFCW-B, SFCW-C and SFCW-Control, respectively. As previously mentioned, the SFCWs could supply an ecological environment for *T. tubifex*. The NH_4-N excreted by *T. tubifex* in its faeces settled to the sediment and could absorbed by *T. orientalis* (Coban et al., 2015b). Meanwhile, in addition to the O_2 input through photosynthesis and ROL by *T. orientalis*, *T. tubifex* could also obtain O_2 through digging networks of galleries and creating biogenic structures (Fig. S2 (Michaud et al., 2005)). Thus, *T. tubifex* can significantly enhance the inorganic N-transformation processes through NH_4-N oxidization in superficial sediment. As a result, the lower NO_3-N and NH_4-N concentration in wastewater resulted in the highest N-removal efficiency for the SFCW-A.

As the main removal pathway, contents of inorganic N in the sediment and *T. orientalis* were also tested in this study (Fig. 2). The inorganic contents of N in sediment achieved stability after 30 days retention, even in the control group. The inorganic N contents in the sediment for SFCW-A were 0.0519±0.0039% and 0.0105±0.0016% for the previous 30 days and later 60 days. And SFCW-C has 0.0415±0.0007% and 0.0095±0.0023% N contents in sediment for the previous 30 days and later 60 days. Compared with SFCW-A and SFCW-C, SFCW-B demonstrated lower inorganic N contents in sediment, with the contents of 0.0366±0.0075% and 0.0052±0.0022% for these two period, respectively. The higher contents of inorganic N with *T. tubifex* in sediment are due to: 1) the direct excretion of *T. tubifex* and later precipitation, leading to NH_4-N in wastewater fluxed to sediment; 2) galleries site at the superficial sediment generated by the bioturbation and burrowing activity by *T. tubifex*, entraining fresh nutrient bound to the sediment and causing the renewal of adsorption (Fig. S2); and 3) the stimulation of microbial activity by *T. tubifex* was significant for N redistribution and transportation, thereby influencing the N storage in the sediment (Fig. S3) (Anschutz et al., 2000; Nogaro & Burgin, 2014). The N deposited in sediment was absorbed by *T. orientalis* as fertilizer. The mass fractions of N storage in the *T. orientalis* for SFCW-A and SFCW-B were depicted in Fig. 2b. Results elucidated that SFCW-A had slightly higher N content than SFCW-B in both belowground and aboveground parts ($p > 0.05$), 3.87% and 22.04% higher, respectively. Thus, *T. tubifex* was conducive to improving N-absorption by plants.

With similar COD concentration in wastewater, the organic carbon in sediment (SOC) for each group, as an electron donor for NO_3-N removal, was also studied. Fig. 2c illustrates the contents of SOC storage in each microcosm. The measured peak contents of SOC were in SFCW-A and SFCW-C, suggesting dramatic increase in SOC as a result of the addition of *T. tubifex*. Due to the presence of *T. tubifex*, electron acceptors (such as O_2 and NO_3-N) for microbes could redistribute in the sediment level. The exchanged redox conditions affected the microbial activity, inducing insufficient degradation of C (Nascimento et al., 2012). As shown in Fig. 2d, the content of organic carbon in *T. orientalis* was not significantly different for SFCW-A and SFCW-B ($p > 0.05$) because carbon in plants is taken up from the air during photosynthesis. Further investigations of CWs should be undertaken for the effect of *T. tubifex* on organic carbon.

Being the main contributor for N-reduction, *T. tubifex* played important role in the microbial abundance and community through their extensive bioturbation activities. The qPCR data showed that *T. tubifex* could slightly increase the absolute abundance of bacterial 16S rRNA ($p > 0.05$, Fig. S4d), indicating its stimulation of microbes. Table 1 gives the relative abundance of related genes for each group. Although *T. tubifex* had a significant effect on common contaminant purification, the measured related microbial abundance showed no significant difference between each group ($p > 0.05$). Since the performance of biological wastewater treatment processes significant depends on microbial community structure, the effect of *T. tubifex* on microbial community may be important for N transformation.

To investigate the abundance and diversity of the bacterial community for each group, Illumina high-throughput sequencing was adopted. In total, 137,823 high-quality reads for the four samples of each group were obtained, with an average read length of 408 bp to 416 bp. Furthermore, the number of OTUs, Good's coverage, Shannon diversity index, Chao and ACE species richness, and Simpson species diversity, at a cutoff level of 3%, were calculated for these samples (Table 2). Based on these indices, the microbial diversity dropped slightly with *T. tubifex*, which was mainly owning to that the increased N with *T. tubifex* may alter the microbial community structure and diversity and subsequent ecological function (Wang et al., 2015b).

The obvious different bacterial composition at the phylum level was also observed, as shown in Fig. 3. *Proteobacteria* was the dominant phylum in each group (with the relative abundance of 36.45%, 41.86%, 42.65% and 42.12%, respectively, for SFCW-A, SFCW-B, SFCW-C and SFCW-Control), followed by *Actinobacteria* (24.98%, 15.89%, 16.31% and 13.00%, respectively), *Bacteroidetes* (17.06%, 6.20%, 22.22% and 8.91%, respectively) and *Cyanobacteria* (19.36%, 10.06%, 3.95% and 5.33%, respectively). Although the bacterial community composition showed similarities, the relative sequence abundance of some phyla varied with the *T. tubifex* addition. Taking *Bacteroidetes* as an example, its relative abundance in SFCW-A and SFCW-C was greatly improved (10.86% and 13.30% higher than that in SFCW-B and SFCW-Control, respectively). The relative abundances of *Acidobacteria*, *Cyanobacteria, Nitrospirae*, *Firmicutes* and *Actinobacteria* were also affected by *T.*

tubifex, which played important roles in N and C cycling (Cydzik-Kwiatkowska, 2015).

It has been reported that the three typical phyla of *Bacteroidetes*, *Proteobacteria* and *Firmicutes* strains is crucial for denitrification process (Miao et al., 2015). These phyla include high levels of bacterial metabolic diversity and many types of denitrifiers. Due to the possible impact of bioturbation activity and pollutant transformation of *T. tubifex* on community abundance, as well as the key bacterial group in wastewater treatment processes, subdivisions of these three phyla were all detected, as shown in Fig. 4. With the total abundance of *Bacteroidetes* in CWs, compared with SFCW-B and SFCW-Control, the *Flavobacteriia* increased by 7.65% and 4.64% in SFCW-A and SFCW-C (Fig. 4a), respectively. It is reported that the *Flavobacteriia* is capable of NO_3-N removal and organic matters degradation, indicating the effect of *T. tubifex* on the anaerobic process (Chen et al., 2015). *Proteobacteria*, the major phylum in CWs, were also found to be sensitive to *T. tubifex* addition. Further analysis under class level revealed that the relative abundances of gamma-*Proteobacteria* were 26.32% and 33.89% for SFCW-A and SFCW-C, which were 8.62% and 13.76% higher than SFCW-B and SFCW-Control, respectively (Fig. 4b). The changes in this abundance were likely responsible for the enhanced denitrification in the microcosms. The alpha- and beta-*Proteobacteria* were also important for organic matter degradation, but showed no significant difference among the groups (Miao et al., 2015). *Firmicutes* is reportedly another key bacterial group in wastewater treatment that inhabits anaerobic environments and withstands harsh environment conditions. As shown in Fig. 4c, the percentage of subdivision *Clostridia* had an apparent increase by 50.16% and 29.12% in SFCW-A and SFCW-C, respectively, which also suggested the enhanced denitrification performance (Wang et al., 2015a). However, *Bacillus*, which is involved in nitrification-aerobic denitrification, showed a slight decrease with *T. tubifex* addition, relating to the lower oxygen level. Overall, the activities of *T. tubifex* greatly stimulate microbial denitrification activities. The lower DO concentration at water-sediment interface and higher carbon source with *T. tubifex* were both important for increased relative abundance of denitrifiers. Furthermore, the microbes ingested by *T. tubifex* keep metabolizing in anaerobic microenvironment in the gut passage, which could induce denitrification activity (Stief, 2013).

In particular, the enhanced TN removal in CWs with *T. tubifex* was highly related to the efficient NO_3-N and NH_4-N removals influenced by *T. tubifex* in wastewater, the higher contents of N adsorbed by sediment and absorbed by *T. orientalis*, and microbial degradation, especially denitrification process.

3.2 Phosphorus removal performance in SFCWs

Some previous studies reported that *T. tubifex* resulted in the increase of P concentrations during wastewater treatment (Hendrickx et al., 2011; Wei et al., 2009). In contrast, this study found that *T. tubifex* could improve P-removal in the overlying wastewater. As shown in Fig. 5a, the TP concentrations in SFCW-A were slightly lower than that of SFCW-B ($p > 0.05$), with the effluent concentrations less than 0.2 mg/L and the removal efficiency up to 70.49±7.60%. The removal efficiency of P in SFCW-B was 43.14±7.68%, with the average effluent ranging from 0.25 to 0.37 mg/L. The TP removal efficiency in SFCW-C and SFCW-Control were 45.63±4.45 mg/L and 27.87±5.20 mg/L, respectively. In addition to plant assimilation and substrate accumulation, biological activity by *T. tubifex* and chemical matter (ion salts) were also important for dephosphorization. Because the enhanced P-removal may be partly due to its transformation by *T. tubifex*, the proportions of particulate and colloidal P in the overlying wastewater were determined in each microcosm. As showing in Fig. 5b and Fig. 5c, higher particulate (22.66±4.01%) and colloidal P (20.57±3.44%) proportions were measured in SFCW-A, as well as SFCW-C (21.48±4.15% and 18.88±3.42% for particulate and colloidal P, respectively). For SFCW-B, the values were only 9.16±2.15% and 9.47±1.93%, respectively. As well as SFCW-Control, with the proportions of 6.50±1.64% and 8.28±2.25%, respectively. The reason is that the fraction of dissolved orthophosphate in the overlying water passed undigested through the gut of *T. tubifex*. Then, this dissolved fraction was transformed into particulate parts via the attachment of P to faeces. In addition, bioturbation of *T. tubifex* increased the contact between orthophosphate and metal ions (such as Fe and Ca) at the water-sediment interface, which could increase the colloidal P proportion (Fig. 5c). The colloidal and particulate P was then bound to the substrate, followed by higher P uptake by the roots of plants, which served as an important approach for P migrated from the water compartment.

With the sediment galleries by bio-irrigation and disturbance, the CWs may efficiently retain the suspended or settled particles in sediment. As shown in Fig. 2e, no significant difference of P content in sediment was detected among all groups throughout the experimental period ($p > 0.05$). During the first 50 days, P content for each group dropped to a level lower than that in the original sediment, and SFCW-A (0.1626 10^{-4}g/g) had 0.70 10^{-6}g/g lower content of P than that of SFCW-B (0.1696 10^{-4}g/g). In these 50 days, the growth of *T. orienalis* in the SFCWs reached the peak and exhibited effective absorption of nutrients. Furthermore, the enhanced colloidal P by *T. tubifex* could also promote P absorption by plants roots. It is supported by the results shows in Fig. 2f that SFCW-A had 31.96% and 25.90% higher P content than

SFCW-B in the belowground and aboveground parts of *T. orientalis*, respectively. Without *T. orientalis* planted in SFCW-C and SFCW-Control, the P contents in sediment were 0.1925 and 0.1793 10^{-4}g/g during the first 50 days, respectively. In these two groups, P contents in SFCW-C was little higher than SFCW-Control, which was due to the higher settled particulate P with *T. tubifex*. During the final 50 days of the experiment, content of P for each group was slightly higher due to P containing in influent and the poor nutrient absorption capacity of the withered plants, as well as the saturation adsorption of sediment. During this experiment period, compared with SFCW-B, SFCW-A had 0.23 10^{-6}g/g higher contents of P because of the metabolisms of *T. tubifex* and the enhancement of particulate P settled in sediment. As well as SFCW-C, which has 0.72 10^{-6}g/g higher P than SFCW-Control. The channel generated by *T. tubifex* could store precipitated particulates. Thus, the residual soluble and particulate P compounds in SFCW-A and SFCW-C were better maintained in the sediment surface than those without *T. tubifex*.

The above results indicated that *T. tubifex* could reduce the P concentration in wastewater through the transformation of P, and further promoting P deposited into sediment and absorbed by *T. orientalis*.

3.3 The role of *T. tubifex* in nutrient removal mechanisms in SFCWs

Based on the above-discussed results, the addition of *T. tubifex* in SFCW systems significantly improved the nutrient removal. Firstly, the activity of *T. tubifex* promoted contaminant transformation in wastewater. The respiration and bioturbation of *T. tubifex* decreased DO concentration at the water-sediment interface, which was beneficial for NO_3-N removal. Meanwhile, their ingestion, extraction and bioturbation increased proportions of particulate and colloidal P in wastewater. Secondly, the bioturbation and digging activity of *T. tubifex* generated galleries at the superficial sediment. The extracted NH_4-N, higher particulate and colloidal P were well deposited in the sediment, and absorbed by the root of *T. orientalis* as a fertilizer. Thus, *T. tubifex* assisted in facilitating the contaminant fluxed from wastewater to sediment. Thirdly, *T. tubifex* positively altered microbial abundance and community structure and organic carbon degradation and further improved denitrification. Due to the sufficient carbon source, lower DO at the water-sediment interface, as well as the anaerobic environment in the gut of *T. tubifex*, the relative abundance of denitrifiers was increased. Thus, the addition of *T. tubifex* in SFCWs could significantly enhance nutrient removal.

4. Conclusions

The removal efficiencies of TN and TP were highly enhanced with the coexistence of *T. orientalis* absorption and biological effect of *T. tubifex*. Lower effluent concentrations of NO_3-N and NH_4-N, higher N storage in sediment and *T. orientalis*, as well as the enhanced microbes relevant to N-transformation contributed to the enhanced N removal with *T. tubifex*. The increased proportions of particulate and colloidal P in effluent related to the enhanced TP removal with *T. tubifex*. The outcomes from this study indicated the highly improved performance of SFCWs with the integrated *T. orientalis-T. Tubifex*-substrate-microbes, thereby making it suitable for application.

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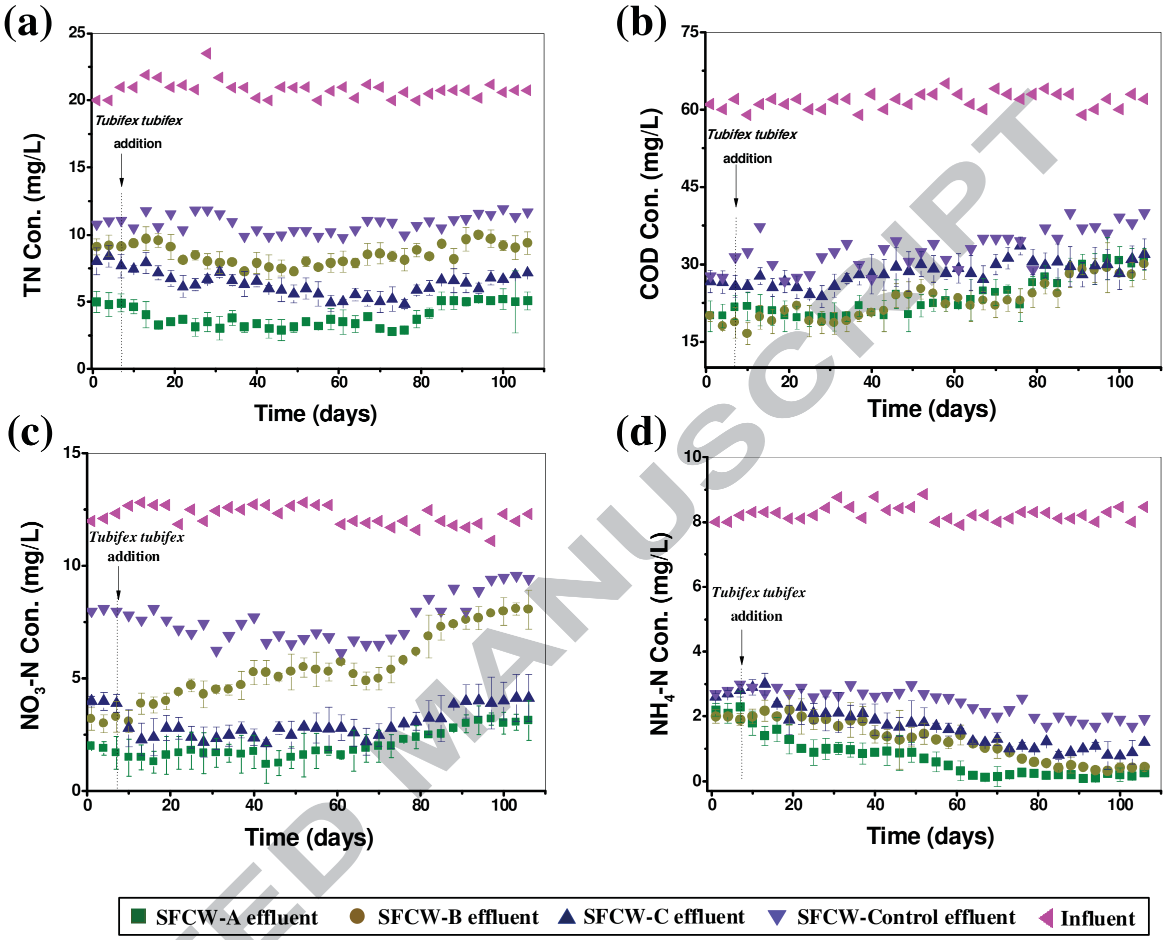


Fig. 1 Treatment performance on TN, COD, NO_3-N and NH_4-N for each group. (a) Influent and effluent of TN concentration for each group, (b) influent and effluent of COD concentration for each group, (c) influent and effluent of NO_3-N concentration for each group, (d) influent and effluent of NH_4-N concentration for each group.

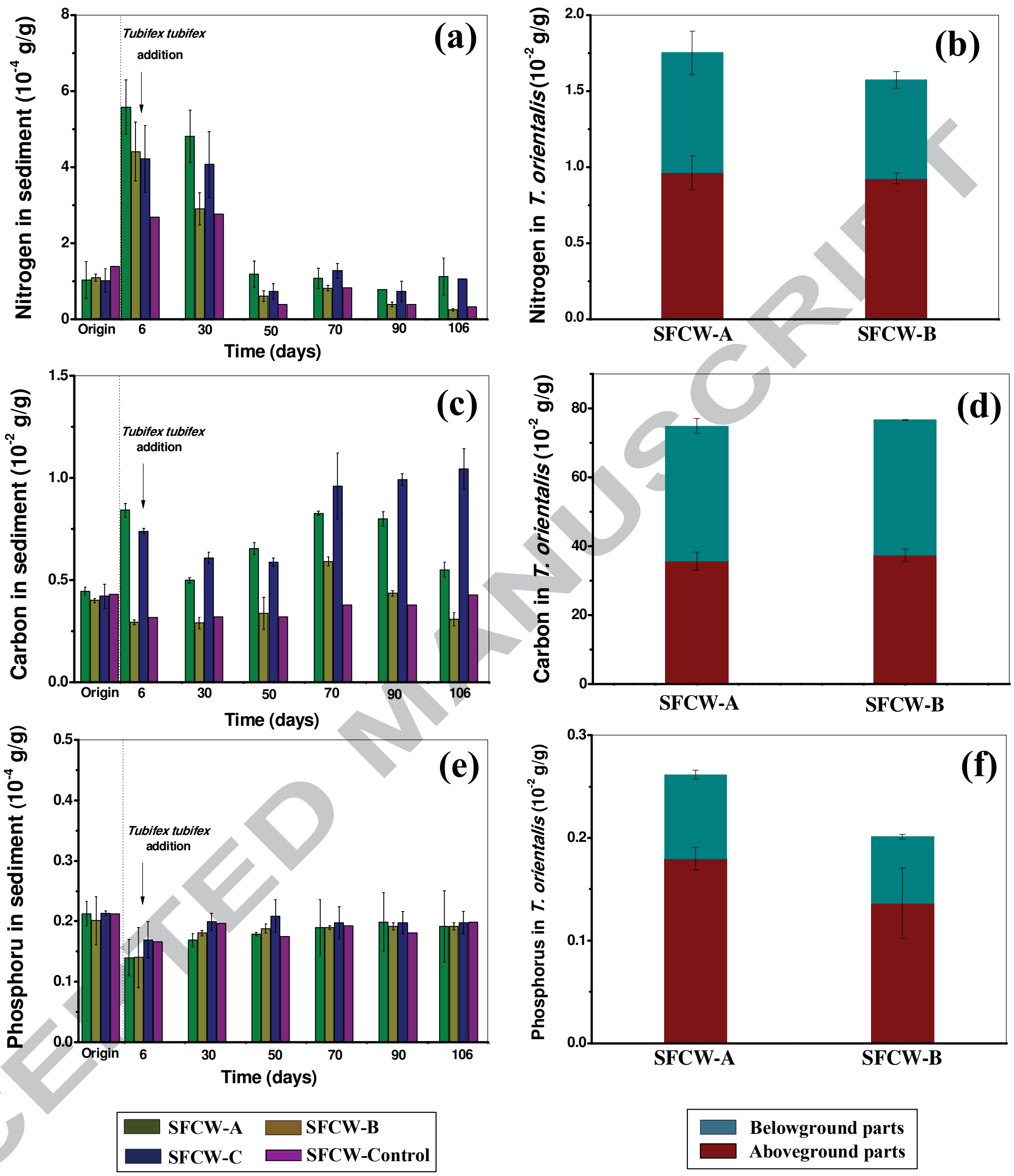


Fig. 2 Treatment performance on N, organic carbon and P storage in sediment and *T. orientalis*. (a) N storage in sediment for each group, (b) N storage in belowground and aboveground parts of *T. orientalis* for SFCW-A and SFCW-B, (c) organic carbon storage in sediment for each group, (d) carbon storage in belowground and aboveground parts of *T. orientalis* for SFCW-A and SFCW-B, (e) P storage in

sediment for each group, (f) P storage in belowground and aboveground parts of *T. orientalis* for SFCW-A and SFCW-B.

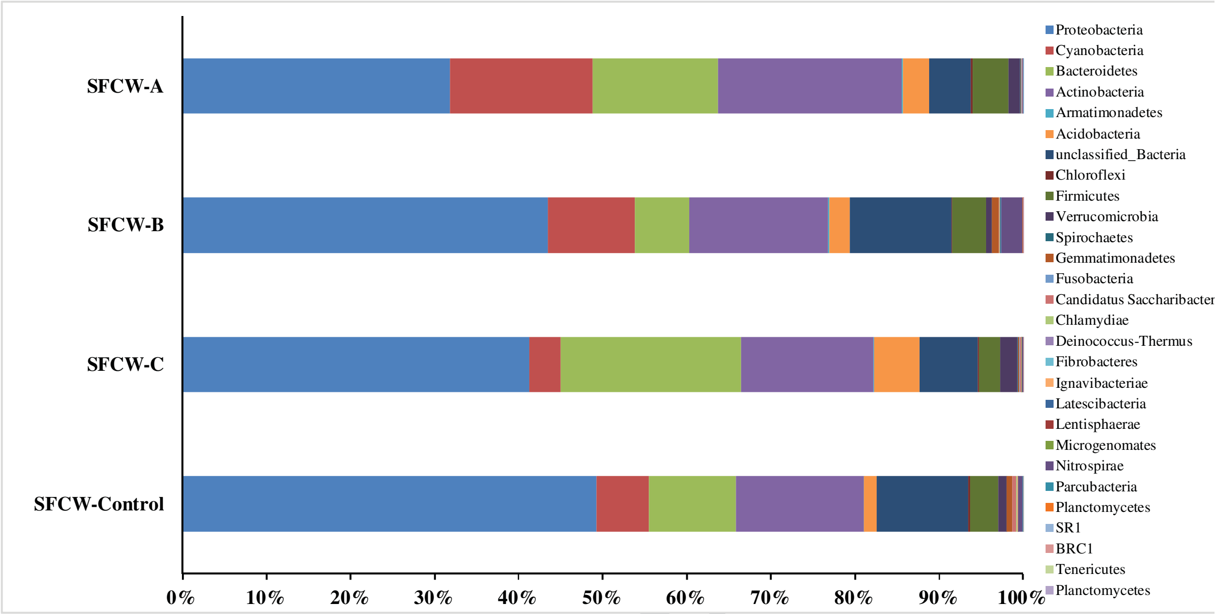
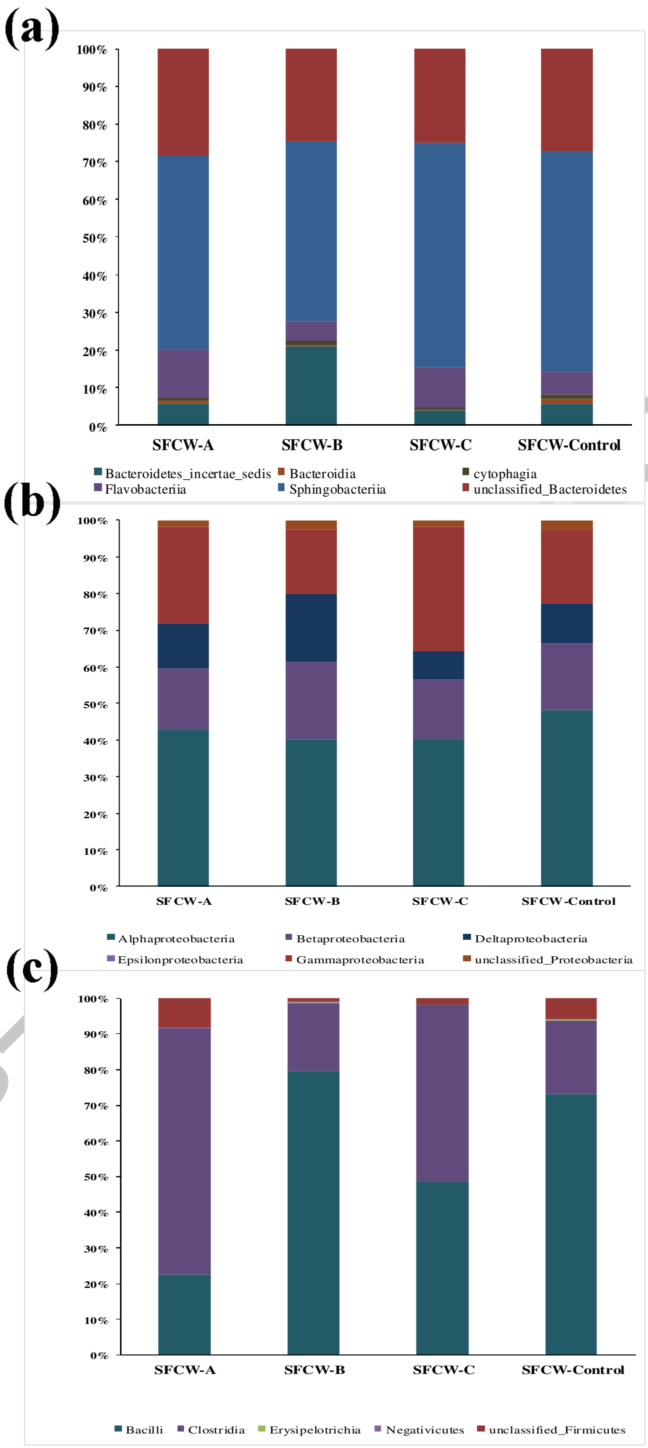


Fig. 3 Bacterial community composition at phylum level as revealed by high-throughput sequencing analyses in SFCWs. Sequences that could not be classified into any known group were assigned as unclassified bacteria.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SFCW-A
SFCW-B
SFCW-C
SFCW-Control
Bacteroidetes_incertae_sedis
Bacteroidia
cytophagia
Flavobacteriia
Sphingobacteriia
unclassified_Bacteroidetes
(b)
100%
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50%
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30%
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SFCW-A
SFCW-B
SFCW-C
SFCW-Control
Alphaproteobacteria
Betaproteobacteria
Deltaproteobacteria
Epsilonproteobacteria
Gammaproteobacteria
unclassified_Proteobacteria
(c)
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50%
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30%
20%
10%
0%
SFCW-A
SFCW-B
SFCW-C
SFCW-Control
Bacilli
Clostridia
Erysipelotrichia
Negativicutes
unclassified_Firmicutes

Fig. 4 Relative abundance of *Bacteroidetes*, *Proteobacteria* and *Firmicutes* subdivisions in SFCWs at class level (a: *Bacteroidetes*; b: *Proteobacteria*; c: *Firmicutes*)

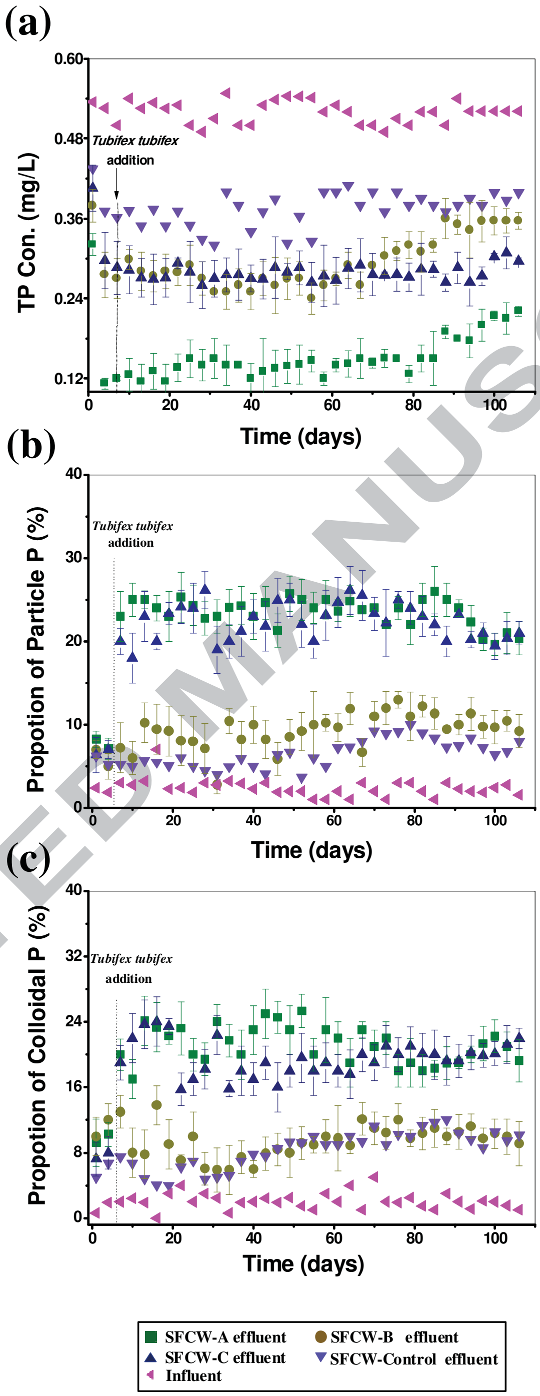


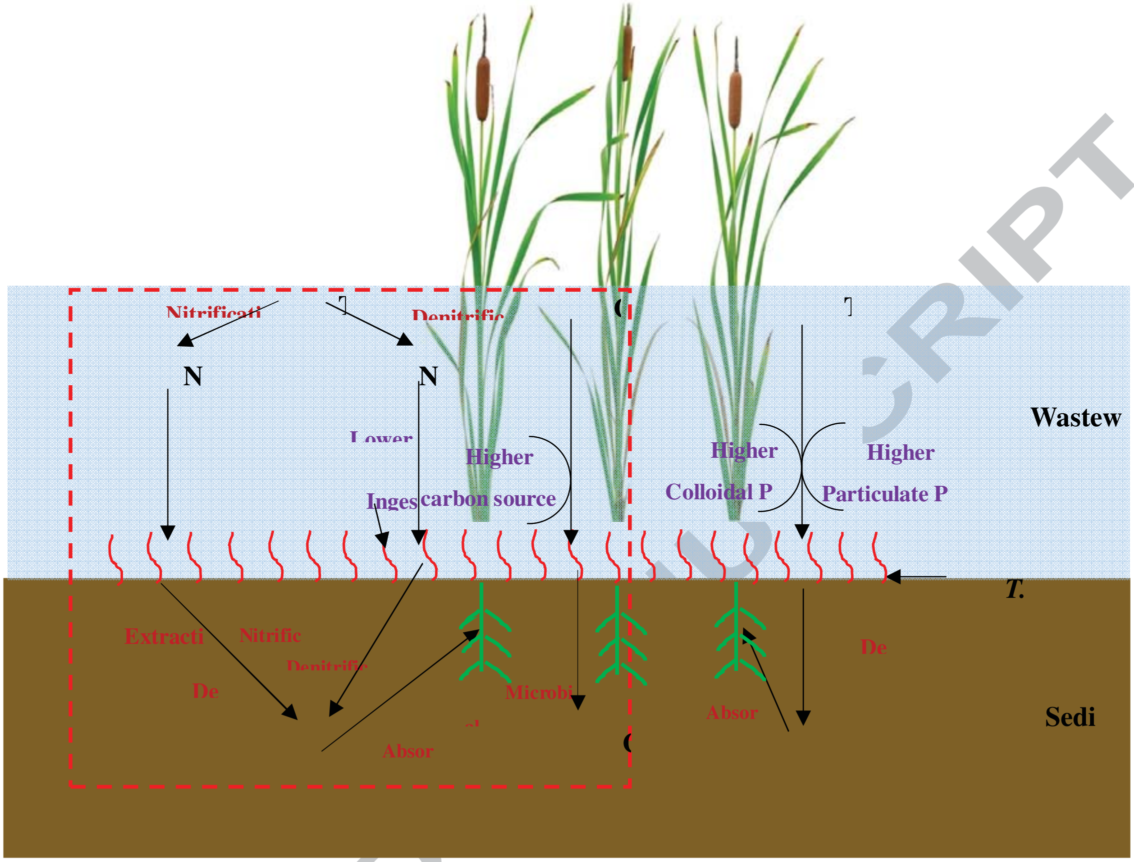
Fig. 5 Influent and effluent TP concentration and proportions of particle and colloidal P. (a) Influent and effluent of TP concentration for each group, (b) influent and effluent proportion of particle P for each group, (c) influent and effluent proportion of colloidal P for each group.

Table 1. Relative abundance of related genes for each group.

Group	amoA (%)	nxrA (%)	nirS (%)	nirK (%)	amx 16S rRNA (%)	nrfA (%)
SFCW-A	0.0098 ±0.0007	0.9288 ±0.0161	5.3027 ±0.5713	1.5924 ±0.3332	6.7052 ±0.8639	6.4164 ±0.6971
SFCW-B	0.0161 ±0.0009	1.5425 ±0.1723	3.6971 ±0.5378	1.1054 ±0.1546	8.7274 ±0.2380	8.7527 ±1.1782
SFCW-C	0.0048 ±0.0010	0.9984 ±0.0249	5.6971 ±0.3676	1.1336 ±0.2712	6.2258 ±0.5645	7.4262 ±0.9269
SFCW-Control	0.0141 ±0.0000	1.2284 ±0.0000	3.6418 ±0.0000	1.1200 ±0.0000	7.4696 ±0.0000	7.3939 ±0.0000

Table 2 Comparison of phylotype coverage, diversity and richness estimators at a phylogenetic distance of 3%.

Sample	OTUs	ACE	Chao	Shannon	Simpson ($\times 10^{-2}$)	Good's coverage (%)
SFCW-A	2634	10519.71	6725.62	5.28	2.54	96.19
SFCW-B	4081	12000.99	8523.04	6.21	0.98	94.54
SFCW-C	2668	9857.91	6462.59	5.46	1.51	95.27
SFCW-Control	3168	17515.9	10066.89	6.12	0.76	91.38


Nitrificati
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Lower
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- The integrated *T. orientalis-T. Tubifex*-substrate-microbes SFCWs is investigated
- The *T. Tubifex* added SFCWs significantly enhance TN and TP removal from wastewater
- Bioturbation with *T. tubifex* provides sufficient carbon source for denitrification
- Increased particulate and colloidal P by *T. tubifex* promoting P removal in SFCWs